Cell mediated immunity in cutaneous infections with human papillomavirus

Thesis submitted for the degree of Doctor of Philosophy by

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Declaration

The studies presented here are the result of my own independent investigation or were performed by others directly under my supervision. Tinneke Herremans assisted with the adhesion assay and Bill Neil cut the cryostat sections and performed the flow cytometry .

This work has not been submitted for candidature for any other degree.

Melany Jackson

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Abstract

Human papillomavirus (HPV) infection of the skin or mucosal surfaces leads to the formation of warts or papillomas. Cutaneous warts frequently persist for several months, even in immunocompetent individuals, suggesting that the virus has developed strategies to evade the normal immune responses of the host. Most evidence indicates that cell mediated immunity is more important than humoral immunity in controlling the extent of HPV infection and its resolution. This study investigated systemic and local cell mediated immunity to HPV in patients with cutaneous warts in order to understand the basis for their persistence or regression.

The aim of the first part of the project was to examine HPV-specific lymphoproliferative responses from the peripheral blood of patients with cutaneous HPV infection. Proliferative T cell responses towards purified HPV *in vitro* were demonstrated in some individuals but frequently responses were weak or non existent. Patients whose warts were regressing (or had fully resolved) did not show increased lymphoproliferative responses towards purified HPV *in vitro* compared with patients whose warts showed no change. HPV-specific T cell clones were not obtained either directly from peripheral blood mononuclear cells stimulated with purified HPV, or after expansion of these cells with anti-CD3 and anti-CD28 antibodies.

Any HPV-specific modulation of immunity may be more apparent locally in the lesion, than in systemic circulation. Therefore three studies were conducted in order to investigate local immunity in cutaneous warts. In the first, an immunohistochemical approach was taken. The density of Langerhans' cells was reduced in the epidermis of warts compared with normal skin. Epidermal keratinocytes did not express intercellular adhesion molecule-1 (ICAM-1) in cutaneous warts, but the vascular expression of ICAM-1 and E-selectin was increased in the dermis of warts compared with normal skin. This may account in part for the observed increase in numbers of T cells in the dermis of warts compared

with normal skin. However, few T cells were found in the epidermis of warts, compared with an inflammatory skin disease such as psoriasis. The second approach was to use a frozen section adhesion assay to investigate functional properties of adhesion molecules expressed in cutaneous warts. It was found that activated T cells bound to wart sections in numbers similar to those bound to sections of normal skin. Reduced numbers of Langerhans' cells and the lack of T cell trafficking into the epidermis of cutaneous warts may be due to local cytokine modulation by HPV. In the third study therefore, mRNA expression of granulocyte macrophage colony stimulating factor, tumour necrosis factor-α, transforming growth factor-β, interleukin (IL)-1α, IL-1β, IL-4, IL-8, IL-10, IL-12 p40, IL-1 receptor antagonist, γ-interferon and the keratinocyte growth factor, amphiregulin, was compared in warts and normal skin. Semi-quantitative analysis by reverse transcription polymerase chain reaction showed significantly increased levels of IL-1α mRNA in warts compared with normal skin, while IL-10 mRNA was significantly reduced in cutaneous warts compared with normal skin. Induced IL-1α expression was found to correlate with amphiregulin mRNA expression within cutaneous warts, which may account for the hyperproliferative nature of these lesions.

Finally preliminary *in vitro* experiments modelling the early stages of HPV infection of keratinocytes were initiated to test whether HPV directly modulates the expression of cytokine mRNA in infected keratinocytes. Although no conclusion was reached, these experiments have highlighted the need for further study.

List of Abbreviations

aa	-	amino acid
APC	-	antigen presenting cell
AR	-	amphiregulin
β-gal	-	beta-galactosidase
BPV	-	bovine papillomavirus
BSA	-	bovine serum albumin
cDNA	-	complimentary deoxyribonucleic acid
CLA	-	cutaneous lymphocyte antigen
CMI	-	cell mediated immunity
COPV	-	canine oral papillomavirus
CRPV	-	cottontail rabbit papillomavirus
CSF	-	colony stimulating factor
CTL	-	cytotoxic T cell
dATP	-	deoxyadenosine triphosphate
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleotide triphosphate
DPV	-	deer papillomavirus
DTT	-	dithiothreitol
EDTA	-	ethylene diamine tetra acetic acid
EGF	-	epidermal growth factor
ELISA	-	enzyme linked immuno sorbent assay
EPV	-	elk papillomavirus
EV	-	epidermodysplasia verruciformis
FITC	-	fluorescein isothiocyanate
γ-IFN	-	gamma interferon
G3PDH	-	glyceraldehyde-3-phosphate dehydrogenase
GM	-	growth medium
GM-CSF	-	granulocyte macrophage colony stimulating factor
HLA	-	human leucocyte antigen
HPV	-	human papillomavirus
ICAM-1	-	intercellular adhesion molecule-1
icIL-1ra	-	intracellular interleukin -1 receptor antagonist
Ii-CS	-	invariant chain
IL	-	interleukin
IL-1R	-	interleukin-1 receptor
IL-1ra	-	interleukin-1 receptor antagonist

IU	-	international units
LFA-1	-	leucocyte function associated protein-1
MadCAM-1	-	mucosal addressin cell adhesion molecule-1
MCP-1	-	monocyte chemotactic protein-1
MEM	-	minimal essential medium
MHC	-	major histocompatability complex
MIP-1β	-	macrophage inflammatory protein-1β
J	-	joules
mRNA	-	messenger ribonucleic acid
NF-1	-	nuclear factor-1
NK	-	natural killer cell
OD	-	optical density
ORF	-	open reading frame
PBMC	-	peripheral blood mononuclear cells
PBS	-	phosphate buffered saline
PCPV	-	pygmy chimpanzee papilloma virus
PCR	-	polymerase chain reaction
PHA	-	phytohaemagglutinin
PMA	-	phorbol myristate acetate
PNad	-	peripheral lymph node addressin
PNK	-	poly nucleotide kinase
PV	-	papillomavirus
RANTES	-	regulated on activation, normal T expressed and secreted
RAR	-	renal allograft recipient
Rb	-	retinoblastoma gene product
RNA	-	ribonucleic acid
RNAsein	-	RNAase inhibitor
RPV	-	rhesus monkey papillomavirus
RT	-	reverse transcriptase
RT-PCR	-	reverse transcription polymerase chain reaction
secIL-1ra	-	secreted interleukin-1 receptor antagonist
SEM	-	standard error of the mean
SSC	-	sodium saline citrate
TAP-1	-	transporter associated with presentation
Taq	-	*Thermus aquaticus* thermostable DNA polymerase
TBS	-	tris buffered saline
TCR	-	T cell receptor
TGF-β	-	transforming growth factor-beta

T_H	-	T helper cell
T_H1	-	T helper type-1
T_H2	-	T helper type-2
TNF-α	-	tumour necrosis factor alpha
UVB	-	ultraviolet B
VCAM-1	-	vascular cell adhesion molecule-1
VLA-4	-	very late antigen-4
VLP	-	virus like particle

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Chapter 1

Introduction

The following study describes an investigation into the role of cell mediated immunity (CMI) in human papillomavirus (HPV) infections of the skin. The introduction outlines the skin immune system, HPV structure and function, natural infection, and CMI towards HPV. A more detailed discussion of the literature relevant to particular aspects of the practical work appears in each experimental chapter.

1.1 The skin immune system

Skin was originally considered to function purely as a physical barrier; however it is now known to be a highly immunoreactive organ, which defends the body from invading pathogens. The skin and its associated lymphoid tissues (SALT) were first described nearly 20 years ago (Streilein, 1978) and are represented diagramatically in Figure 1.1. The epidermis and dermis are the two principal compartments of the skin and are separated by a basement membrane. Keratinocytes (KC) make up 80% of the cells in the epidermis forming a stratified multilayered epithelium. The KC adjacent to the basement membrane are predominantly undifferentiated rapidly proliferating stem cells. As KC become committed to differentiate, they detach from the basement membrane, lose their capacity to proliferate and migrate through the supra-basal layers towards the stratum corneum. Terminally differentiated KC are finally shed from the stratum corneum as dead cornified squames.

In addition to forming a physical barrier KC have an important immunoregulatory role. KC can be stimulated in culture to secrete many cytokines including interleukin (IL) -1 (Kutsch *et al* 1993), tumour necrosis factor-α (TNF-α), IL-8

(Matsushima *et al* 1989), IL-6 and granulocyte macrophage colony stimulating factor (GM-CSF) (Schrader, 1994). Although cytokines are not expressed constitutively by KC *in vivo* they can be induced by a variety of stimuli. Furthermore, expression of cell surface molecules such as major histocompatibility (MHC) class II antigens or intercellular adhesion molecule-1 (ICAM-1) are induced on KC in inflammatory reactions of the skin (Barker *et al* 1989). MHC class II^+ KC can also present bacterial superantigens, *Mycobacterium leprae* and herpes simplex virus antigens to T cells *in vitro* (Nickoloff *et al* 1993; Mutis *et al* 1993; Cunningham *et al* 1989), thus emphasising the potential role of KC in regulating the skin immune system.

In addition to KC, the other components of the skin include Langerhans' cells (LC), leucocytes, endothelial cells, melanocytes and mast cells. Langerhans' cells are $CD1a^+$, MHC class II^+ dendritic cells derived from the bone marrow which form a semi-continuous network between epidermal KC and are the main antigen presenting cells (APC) of the skin. Unlike KC, LC are a migratory population of cells and do not permanently reside in the epidermis. Immature LC in the epidermis develop the capacity to become potent APC after migration from the skin to the draining lymph node where they process and present antigens to naive T cells (Streilein, 1990). In a secondary response, antigen presentation to memory T cells may then occur within the skin by LC, macrophages or fibroblasts (Streilein, 1983). Invading pathogens, contact sensitisers or ultraviolet B light (UVB) exposure can all induce LC to migrate from the epidermis. TNF-α has been proposed to initiate the movement of LC from the skin to the draining lymph node (Cumberbatch *et al* 1995). Dermal dendrocytes are another population of $CD1a^+$ dendritic cells which are thought to contribute to antigen presentation in the skin (reviewed by Lappin *et al* 1996).

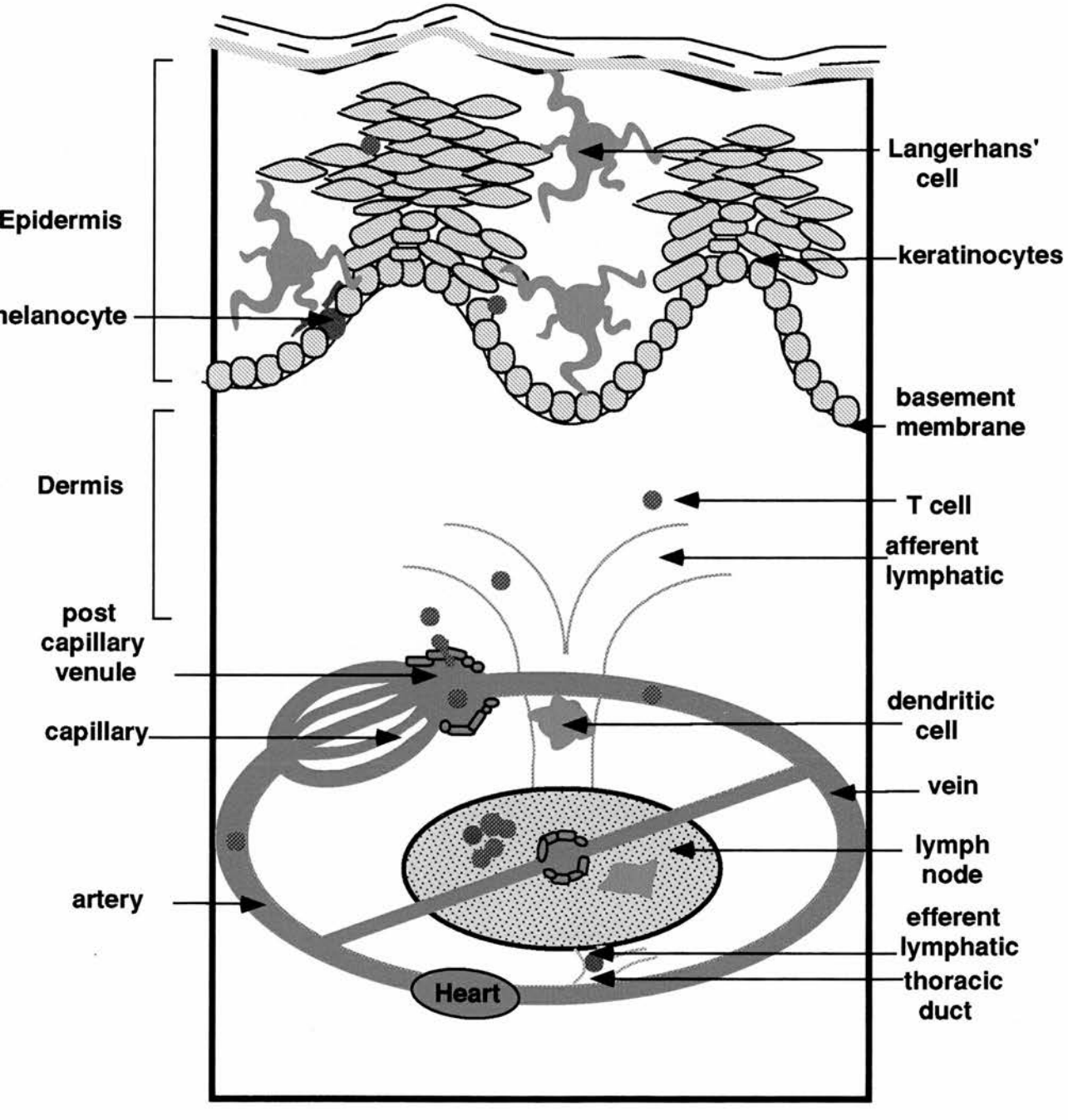
T cells, macrophages and other leucocytes enter the dermal and epidermal

compartments from dermal blood vessels. The majority of T cells within normal skin comprise equal numbers of $CD4^+$ and $CD8^+$ subsets and are located perivascularly in the dermis. In contrast only 2% of the T cells in normal skin are found in the epidermis and are predominantly $CD8^+$ (Bos *et al* 1987). Most cutaneous T cells express α/β T cell receptors (TCR) (Foster *et al* 1990) whereas cells expressing γ/δ are rare. However, a unique population of dendritic epidermal T cells with γ/δ TCR dedicated to epithelial immunosurveillance does exist in rodents (Janeway *et al* 1988), but an equivalent cell population has not been identified in humans.

Endothelial cells make up the dermal blood vasculature and play a central role in cutaneous inflammation. Cytokines such as IL-1 and TNF-α rapidly induce endothelial cells to perform inflammatory functions by the expression of leucocyte adhesion molecules. The cell adhesion molecules ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin differ in their specificity for leucocytes and in the timing of their expression after activation of endothelium. There is also evidence that cutaneous leucocyte antigen (CLA) expressed on $CD4^+$, $CD45RO^+$ T cells acts as a skin-specific homing receptor through its interaction with E-selectin vascular endothelial cells (Berg *et al* 1991; Picker *et al* 1991) (discussed in more detail in chapter 3, section 3.1.1).

Melanocytes are neural crest-derived cells present in the basal layer of the epidermis. Their primary function is to protect the body from UV irradiation by the production and transfer of melanin to KC. When stimulated, melanocytes respond to many cytokines and secrete IL-1α, β, transforming growth factor-β (TGF-β), IL-8 and monocyte chemotactic protein-1 (MCP-1) (reviewed by Armstrong *et al* 1994). In addition ICAM-1 can be induced on the surface of melanocytes, thus promoting cell-cell interactions.

Figure 1.1 Skin associated lymphoid tissues (SALT)



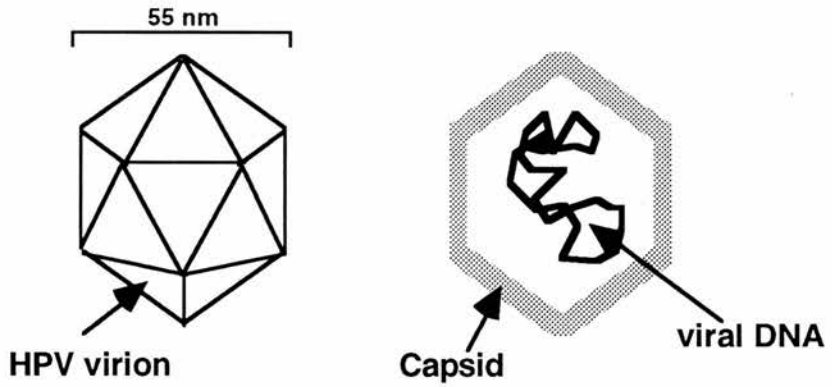
Taken from Streilein J.W. (1989) Skin-associated lymphoid tissues (SALT): the next generation. In: Skin Immune System. Edited by Bos J.D. CRC Press Inc. pp 26-48.

Mast cells are bone marrow derived cells which in skin are located in close proximity to blood vessels but are rarely found in the epidermis. These myeloid cells store preformed enzymes, glycosaminoglycans, mediators of inflammation such as histamine and express the high affinity IgE receptor on their cell surface. The pattern of cytokines synthesised by mast cells is similar to a T helper type 2 (T_H2) response, favouring IgE dependent immunological processes. Mast cells can also synthesise IL-1, 3, 4, 5, 6, 8, TNF-α, TGF-α and GM-CSF and can be found in increased numbers in chronic inflammatory diseases of the skin (reviewed by Moller *et al* 1994).

1.2 Human papillomavirus structure

HPV are icosahedral particles containing a single 8 Kb double stranded circular DNA genome (Figure 1.2) which exists in a chromatin-like complex with cellular histones. A proteinaceous coat, known as the capsid, protects the viral genome and contains determinants which promote adherence of virus particles to receptors on host cells.

Figure 1.2 HPV particle structure



Taken from Lowry *et al* (1991)

1.2.1 Structural proteins

The capsid of each infectious virion is composed of 72 capsomeres comprising the virally encoded major and minor capsid proteins named L1 and L2 respectively

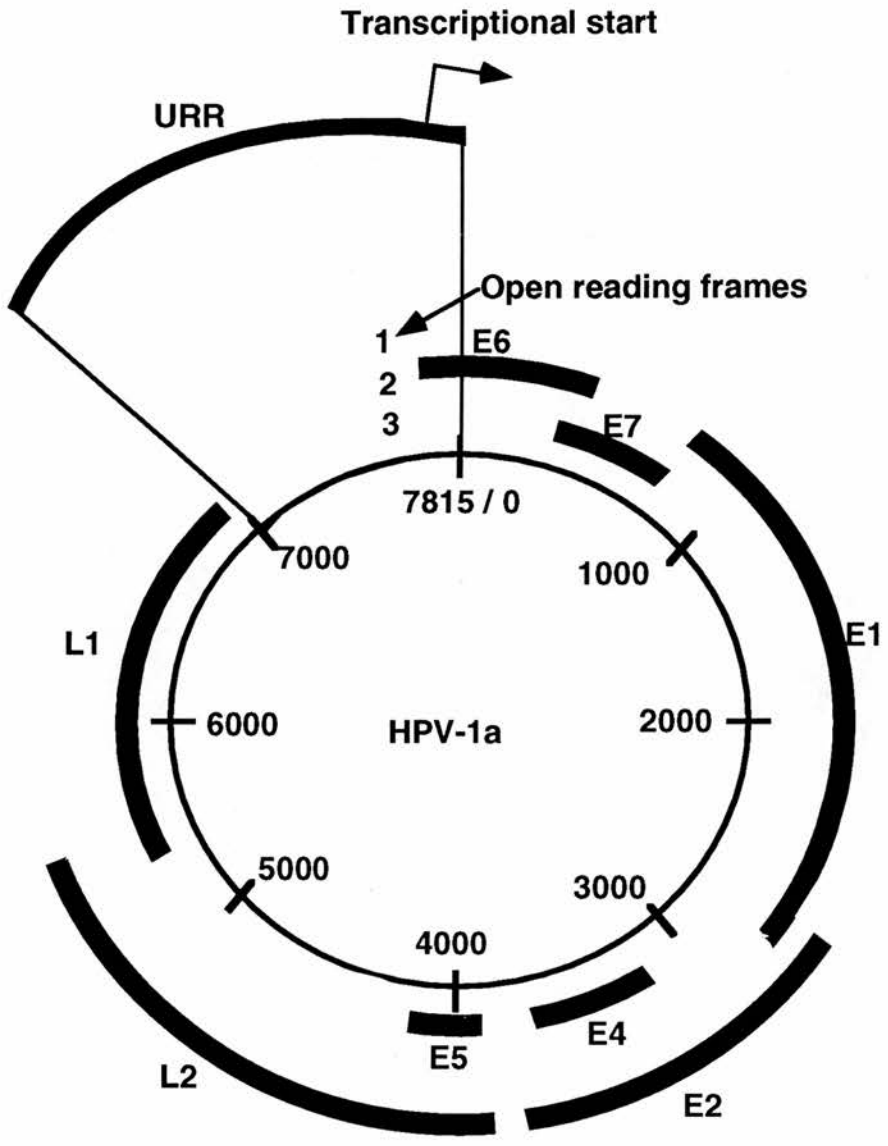
(reviewed by Pfister *et al* 1994). Each capsomere is a pentameric structure, formed predominantly from L1, which has a highly conserved amino acid sequence amongst different HPV types. The amino acid (aa) sequence of L2 is less well conserved between different HPV types and is thought to be type specific.

Studies on the functions of capsid proteins have been facilitated by the discovery that HPV-16 L1 and L2 proteins, when expressed as recombinants in vaccinia virus, self-assemble to form empty virus-like particles (VLPs) (Zhou *et al* 1991 and reviewed by Schiller *et al* 1995). As judged by cryoelectron microscopy HPV-1 VLPs are indistinguishable from whole HPV-1 particles (Hagensee *et al* 1994). L1 protein has been shown to self-assemble into VLPs in all papillomavirus types tested (Schiller *et al* 1995) and self-assembly is an intrinsic property of the L1 protein. The functions of L2 are less clear, since L2 alone cannot self-assemble (Hagensee *et al* 1993) and VLPs containing only L1 appear to be identical to capsids with both L1 and L2 (Hagensee *et al* 1994). However, the presence of L2 increases the efficiency of assembly of L1 (Hagensee *et al* 1993). Interestingly, the amino terminus of L2 can bind non-specifically to DNA, which suggests a role for L2 in virion assembly (Zhou *et al* 1994).

1.2.2 Genomic structure and organisation

The double stranded circular HPV DNA genome is represented in Figure 1.3. Nomenclature of the genes is based upon the time when they are expressed in the viral replication cycle (early or late). In infectious HPV particles the genomic DNA is covalently closed, allowing super helical twists, but when viral DNA is replicating, relaxed or open circular DNA predominates. HPV proteins are expressed from open reading frames (ORFs) located on one DNA strand. The second DNA strand is non-coding, but may have a regulatory role. The upstream regulatory region (URR) contains the promoter elements and varies in size from 500 to 1000 bp depending on HPV type. Transcription factors such as NF-1, AP-1 and

Figure 1.3 Genomic map of HPV-1a



Taken from Giri *et al* 1986. Thick lines show the HPV-1a open reading frames (ORFs). The HPV genome can be divided into 3 domains: early gene products, late gene products and upstream regulatory region (URR) containing binding motifs for regulators of transcription and replication.

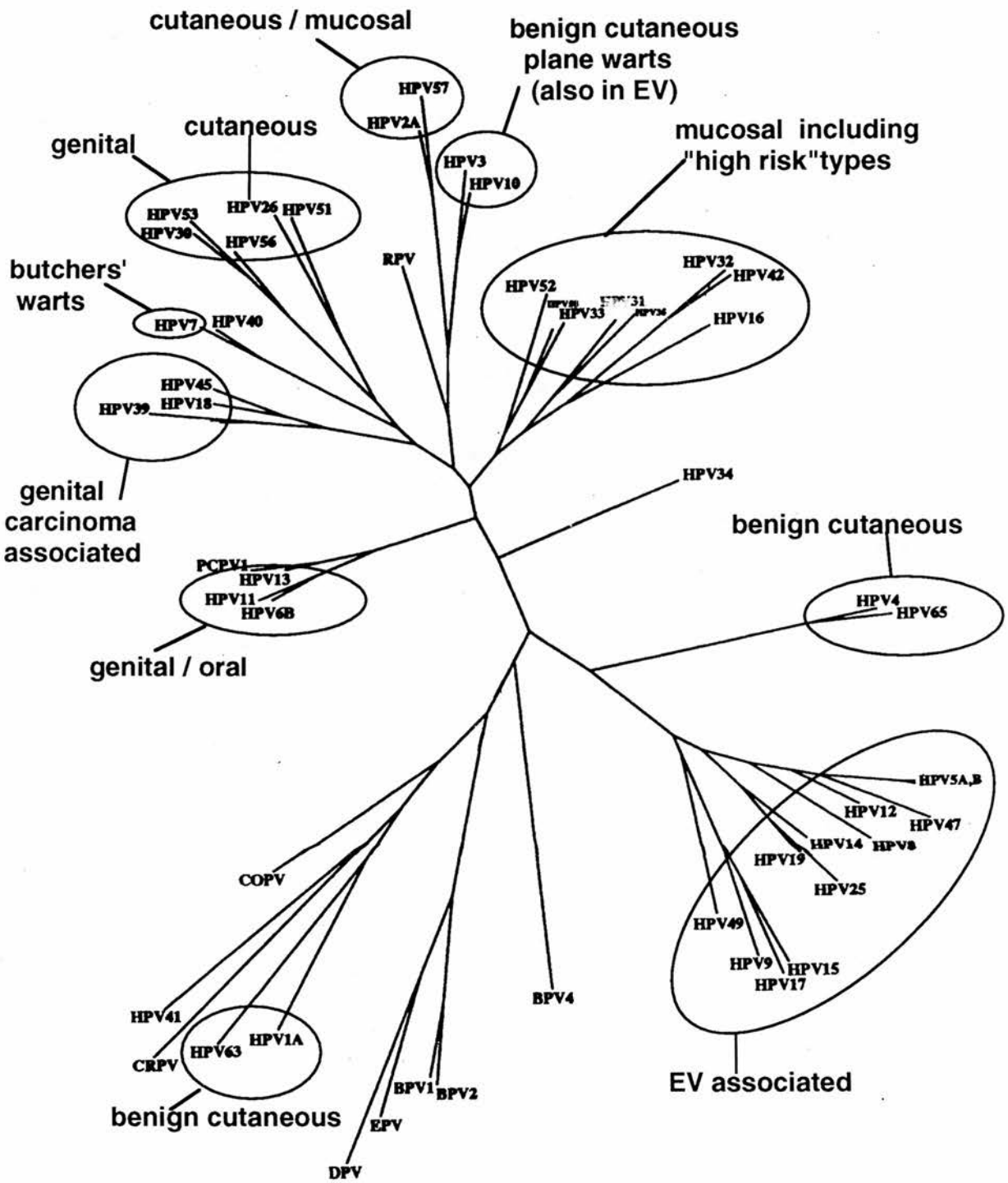
SP-1 bind to specific motifs which are repeated within the URR and regulate gene expression. Response elements for HPV-E2, E6/E7 and the glucocorticoid/progesterone receptors are also present within the URR.

1.3 HPV types.

Papillomaviruses (PV) are species specific and 77 different types which infect humans have been identified to date. Originally a new HPV type was defined by less than 50% cross hybridisation of the viral DNA to all known HPV types under stringent conditions. Using sequence comparison, new types are now assigned if there is < 90% homology in the DNA sequence of the L1, E6 and E7 ORFs to all known types. Homology of >90% designates it a sub type of a known HPV (De Villers, 1994). Figure 1.4 shows a phylogenetic tree of relatedness of different PV types. PV have evolved relatively slowly and as these viruses rarely cross the species barrier, the separation between 2 PV types may have occurred at the same time as the separation between the 2 host species (reviewed by Bernard *et al* 1994). Interestingly HPV-1, 41 and 63 are more closely related to cottontail rabbit PV (CRPV) and canine oral PV (COPV) than to other human PV and pigmy chimpanzee PV (PCPV) shares the greatest sequence homology to HPV-13.

Individual HPV types are associated with infection of either cutaneous or mucosal epithelia (shown in Table 1.1). HPV-1, 2 and 4 are the main aetiological agents of benign cutaneous papillomas (shown in Figure 1.5 and 1.6). However, some HPVs infect both epithelial surfaces; for example, HPV-2a and HPV-57 which are closely related types, infect both mucosal and cutaneous tissue, but HPV-57 preferentially infects mucosal tissue and HPV-2a infects cutaneous tissue (Hirsch-Benham *et al* 1990).

Figure 1.4 Phylogenetic tree of 54 types of papillomavirus



The phylogenetic tree (taken from Bernard *et al* 1994) is based on homology comparisons of two highly conserved segments of the L1 gene with a combined length of 420 bp. DPV = deer PV BPV = bovine PV, CRPV = cottontail rabbit PV, COPV = canine oral PV, RPV = rhesus monkey PV, EPV = elk papillomavirus.

Table 1.1 Clinical associations of different HPV types

Clinical lesions	HPV types
Skin	
Plantar warts	1, 2, 4, 63
Common warts	2, 4, 26, 27, 29, 57, 65
Plane warts	3, 10, 28, 49,
Butchers' warts	7
Benign EV lesions	2, 3, 10, 12, 15, 19, 36, 46, 47, 50
EV benign or malignant	5, 8, 9, 10, 14, 17, 20-25, 37
Non-warty skin lesions	37, 38
Genital	
Condylomata acuminata	6, 11, 42-44
Non condylomatous lesions and/or CIN	6, 11, 16, 18, 30, 31, 33, 34, 35, 39, 40, 42, 43, 51, 52, 55, 56, 57-59, 61, 62, 64
Carcinoma (cervix, penis, vulva)	16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 66
Non-genital mucosal	
Mouth	13, 32
Laryngeal papilloma	6, 11, 30
Maxillary sinus papilloma	57
Carcinoma (head, neck, lung)	2, 6, 11, 16, 18, 30

Taken from (Cossart *et al* 1995). EV = epidermodysplasia verruciformis, CIN = cervical intraepithelial neoplasia.

Figure 1.5 Common hand warts on an immunocompetent individual

Reproduced with kind permission of Dr. E.C. Benton, Department of Dermatology, Royal Infirmary, Edinburgh.

Figure 1.6 Extensive mosaic plantar warts in an immunosuppressed renal allograft recipient

Reproduced with kind permission of Dr. E.C. Benton, Department of Dermatology, Royal Infirmary, Edinburgh.

1.4 Non-structural open reading frame proteins

1.4.1 Papillomavirus E1

E1 protein plays a role in regulating viral DNA replication and is the largest of the early genes expressed in HPV infection (reviewed by Storey, 1994 and Lambert, 1991). Experiments with BPV-1 have shown that a complex of the E1 protein and the E2 protein bind specifically to the origin of replication (Mohr *et al* 1990). Full length E1 and E2 proteins are required for optimal viral replication and may be involved in both positive and negative regulation of this process (reviewed by Cossart *et al* 1995). Although less is known about the HPV E1, the HPV-1a E1 protein alone is sufficient for low levels of DNA replication. The domain of E1 that modulates replication has been mapped to the 5' end of the E1 gene and is termed E1M.

1.4.2 Papillomavirus E2

The E2 ORF codes for the main transcriptional regulatory protein of the virus which also plays a role in DNA replication (Lambert, 1991). The full length E2 protein can trans-activate or repress HPV promoters through binding to E2 responsive enhancer elements which are repeated many times in the URR. Alternative splicing of E2 produces 2 truncated forms of E2 protein that have negative regulatory effects on viral transcription (reviewed by Storey, 1994). E2 proteins consisting predominantly of the carboxyl half (E2C) repress the activity of the full length E2 by competitive binding to the DNA motif, or by forming complexes with E2, thus inhibiting its activity (Lambert, 1991). The activity of E6/E7 promoters in HPV-11, 16, and 18 are inhibited by binding of the transactivating E2 protein. Distance from the DNA bound by the E2 and the transcriptional start may determine whether transcription is stimulated or repressed (reviewed by Cossart *et al* 1995). E1 from HPV-11 in combination with E2 from either HPV-6, 16, or 18 support the replication of HPV-11 genome, indicating that E1 and E2 proteins have shared

functions between different HPV types (Del Vecchio *et al* 1992). E2 also increases the efficiency of binding of the E1protein and thus influences DNA replication (Gopalakrishnan *et al* 1995). An E1M^E2C transcript detected in C127 cells containing HPV-11 codes for an E2 binding site specific transcriptional repressor (Chaing *et al* 1991).

1.4.3 Papillomavirus E4

E4 is highly expressed in palmar and plantar warts induced by HPV-1 infection, and is also found in HPV-2, 4, 11 and 16 infections. Although E4 was originally defined as an early gene it is expressed late in the viral life cycle. E4 is localised in the cytoplasm and does not have any transforming functions. At the amino acid level there is little homology between E4 products from different HPV types. However, E4 proteins are more closely related in HPVs which infect similar epithelial surfaces, suggesting a role for the protein in the epitheliotropism of HPV. Alternate forms of E4 are found in HPV infection, and are differentially expressed during KC differentiation. The primary product of HPV-1 E4 ORF is a 17 kD E1^E4 spliced mRNA which has 5 amino acids from E1 (Chow *et al* 1987). In cells expressing E4, the cytokeratins associated with differentiation (K1 and K10) are absent. This led to the proposal that E4 may replace cytokeratins and is involved in virus release (Doorbar *et al* 1988). Furthermore, E4 interacts with filaggrin, a known cytokeratin binding protein. In papillomas there is a correlation between the amount of virus and E4 expression. For example HPV-1 induced warts have a high viral content and the highest level of E4 protein (reviewed by Doorbar, 1991).

1.4.4 Papillomavirus E5

The E5 ORF is the least conserved region of the HPV genome and can be absent or truncated in many HPV. When present, its expression is localised to the cytoplasmic membrane (reviewed by Storey, 1994). Papillomavirus E5 was initially not thought to play a role in cellular transformation since it is lost from many cervical tumours.

However, BPV-1 E5 efficiently transforms cells when transfected *in vitro* and HPV-16 E5 stimulates the growth of rodent fibroblasts. HPV-16 E5 acts in association with the epidermal growth factor (EGF) receptor to increase the expression of cellular *fos* gene (Leechanachai *et al* 1992). Since, the URR has many *fos/jun* responsive AP-1 sites the E5 protein may indirectly induce viral gene transcription. In cutaneous warts infected with HPV-1, E5 protein is localised to the perinuclear halos of koilocytes (Figure 1.8), but its exact function in papillomas remains undetermined (reviewed by Storey, 1994).

1.4.5 Papillomavirus E6

The E6 ORF is an HPV oncogene which is expressed in cell lines derived from cervical carcinoma even when other HPV genes have been lost. Therefore the continued presence of E6 is required for the maintenance of the transformed phenotype of these cells. HPV-16 and 18 E6 proteins interact with the p53 tumour suppressor gene product and lead to its degradation, thus eliminating the suppressive effects of p53 on cell division. Mutations in p53 are found in many tumours and cause altered cell cycle regulation. E6 protein binds to E6-associated protein, a member of the ubiquitin degradation pathway, necessary for the degradation of p53 (reviewed by Androphy, 1994). Thus, E6 mediated degradation of wild type p53 is functionally analogous to the mutation of wild type p53. HPV-6 and 11 E6 proteins, associated with benign mucosal lesions, "weakly" interact with wild type p53 and fail to promote its degradation *in vitro*. Interestingly, although HPV-1 E6 gene products do not complex with p53 and therefore do not promote its degradation the HPV-1 E6 protein can inhibit the transcriptional regulatory activity of p53 (Kiyono *et al* 1994). However other cutaneous HPV types associated with EV (5, 8, or 47) do not show a similar inhibition of p53 function.

1.4.6 Papillomavirus E7

The E7 ORF is the most widely studied papillomavirus oncogene. In common with

E6, E7 is continually expressed in cervical tumours and cell lines immortalised *in vitro*, and can maintain the transformed phenotype of the cells. E7 protein binds to the retinoblastoma tumour suppresser gene product (Rb) and the related p107 protein, both of which are involved in cell cycle regulation. In G1 of the cell cycle, Rb or p107 are complexed with E2F, a transcription factor. When cells move into S phase, the Rb/p107 is phosphorylated and the Rb-E2F (or p107 /E2F) complex dissociates. E2F is then released and is involved in transactivation of many cellular genes involved in cell division (reviewed by Androphy, 1994). E7 prevents Rb from complexing with E2F and induces cellular proliferation. In an *in vitro* organotypic culture E7 is also capable of reactivating cellular DNA replication in suprabasal KC (Cheng *et al* 1995). Different domains of E7 protein determine trans-activation and transformation. "High risk" HPV-types (HPV-16 and 18) bind Rb or p107 more efficiently than "low risk" (HPV-6 and 11), which may explain the differences between these HPV types in the progression to carcinomas. Cervical tumours lacking HPV sequences often carry mutations in the p53 and Rb genes. In contrast, cervical tumours containing HPV DNA possess wild type Rb and p53 sequences. Cutaneous infections in immunocompetent individuals do not usually progress to carcinomas (Benton *et al* 1992), and the genomes of these cutaneous HPV types do not cause transformation *in vitro*. It remains unknown whether E6 and E7 from cutaneous HPV types have a role in self-limiting hyperproliferation, characteristic of benign papillomas.

1.5 Natural infection with HPV

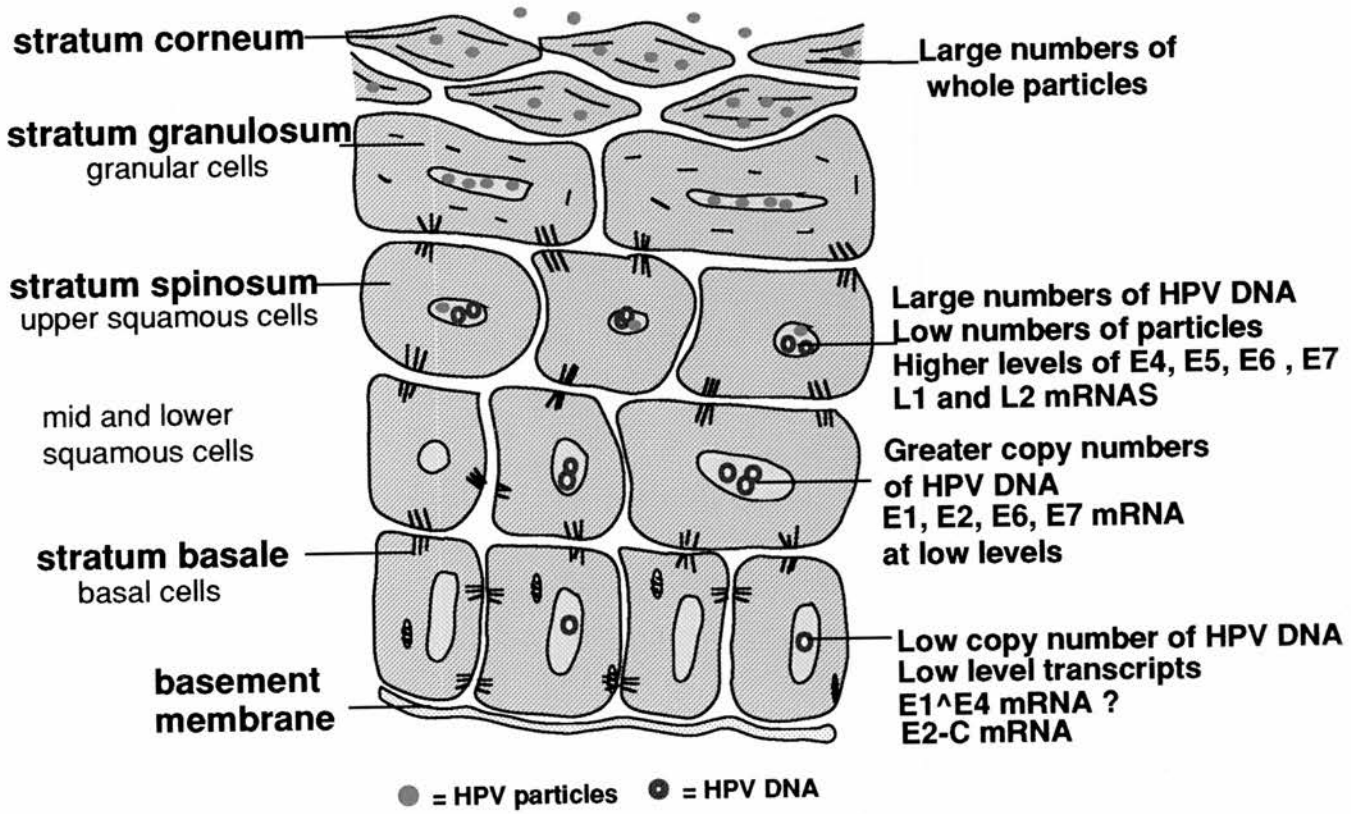
Productive HPV infection is restricted to stratified squamous epithelium and the viral replication is intimately linked to the differentiation of host KC represented diagramatically in Figure 1.7. HPV infection of basal KC is thought to occur via an abrasion or breach into the deeper layers of the epidermis. The virus adsorbs to the plasma membrane of the host cell to gain entry, probably through an interaction of

capsid components with specific receptors on the KC membrane. Although it remains undefined which determinants are involved in the binding of HPV to host cells, studies using VLPs demonstrated the importance of L1 in this process. Capsids containing only L1 were as efficient at binding and penetrating host cells as VLPs containing L1 and L2 (Muller *et al* 1995). Animal and human PVs enter host cells via a similar cell surface receptor (Muller *et al* 1995). Therefore species and epithelial specificity may reside in cellular events later in the infectious cycle. In an *in vitro* system, BPV-1 particles can adsorb to C127 cells in 30 minutes at 37^{o}C and are completely taken up by 90 minutes. Since virus particles were not observed in the nucleus of infected cells uncoating of virions was presumed to occur in the cytoplasm (Zhou *et al* 1995). Indeed other studies provided evidence that following adsorption, the virus penetrates the cytoplasm of the KC by receptor mediated endocytosis (Muller *et al* 1995).

Once inside the cell the virus is disassociated through the separation of the capsid and viral DNA. In benign cutaneous warts and low grade cervical intraepithelial neoplasia, episomal HPV DNA is found in the nucleus of basal cells, where it replicates but remains at low copy number (20 -100 copies / cell). Capsid proteins or whole virus particles are not found in this basal KC layer.

The differentiation of the host cell induces extensive HPV DNA replication (1000s of copies / cell), in addition to the expression of early and late proteins. HPV particles are assembled in the stratum granulosum and many whole virions can be detected in the cornified layers of the wart. HPV virions are released when KC slough off from the highly keratinised stratum corneum. HPV infections which progress to high grade carcinomas, usually contain HPV-DNA integrated into the cellular genome without the production of virions.

Figure 1.7 The relationship between epithelial differentiation and the different stages of HPV replication



Taken from Cossart YE, Thompson C, Rose B. (1995) Virology.
In: Genital warts : Human papillomavirus infection.
Edited by Mindel A. Edward Arnold Publishing. pp 1-34.

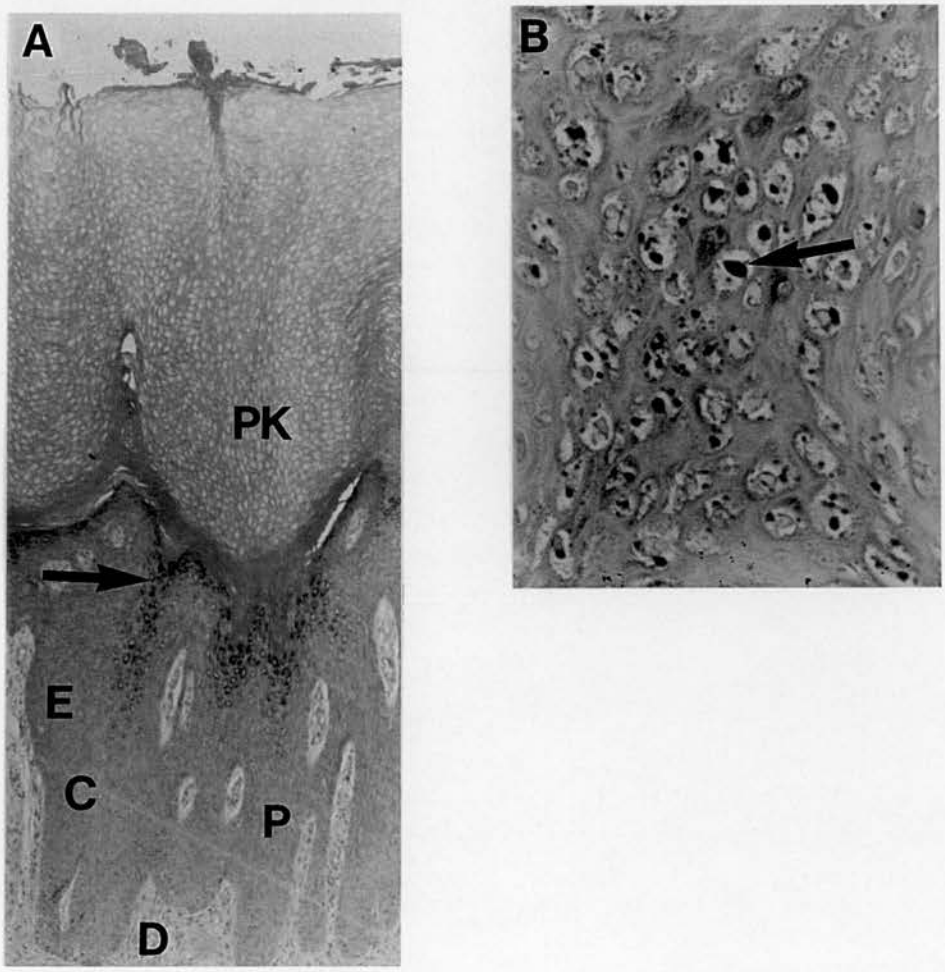
Studies of HPV-11 infection in nude mice, demonstrate transcription of early genes 4 weeks after initial infection (Stoler *et al* 1990). Viral replication and transcription of early and late ORFs occurs maximally at 6-8 weeks, and after 10 weeks condylomata are formed. However, in natural HPV infection, viral DNA may persist in the basal cell layer, thus remaining latent before DNA replication and a productive infection takes place.

Normal epidermis has an ordered keratin expression pattern from basal to terminally differentiated KC that becomes disordered in HPV infection. The cytopathic effect of HPV infection in skin is shown by the presence of koilocytes (large KC with small pyknotic nuclei surrounded by a clear cytoplasm), parakeratosis (where nuclear remnants persist in the superficial keratin layers), hyperkeratosis, (increase in the horny layer), perinuclear vacuolisation, acanthosis (thickening) and papillomatosis (folding) (Bunney *et al* 1992b) and are illustrated in Figure 1.8.

1.6 Prevalence of cutaneous HPV infection

The prevalence of cutaneous warts in the immunocompetent population is difficult to determine accurately since diagnostic methods to detect previous infection are not routine. Commonly used tests to detect HPV DNA in biopsy material are low-stringency Southern blot, PCR with consensus L1 primers, dot blot and hybrid capture. Early studies based on serological tests have estimated the prevalence to be between 7 and 13 % of the population (reviewed by Bunney *et al* 1992a). However, more recent estimations of exposure to HPV using ELISA have shown that 85% of individuals without current cutaneous warts have HPV-1 specific serum antibodies (Steele *et al* 1990).

Figure 1.8 The cytopathic effect of HPV in cutaneous warts



Photomicrographs of cutaneous viral wart sections stained with haematoxylin and eosin are shown. Magnifications are : 12 X (panel A) and 100 X (panel B). Panel A: parakeratosis = PK, acanthosis = C, papillomatosis = P, epidermis = E, dermis = D. The arrow indicates a prominent granular layer with vacuolated cells. Panel B : the arrow indicates koilocytes in the upper epidermis of a palmar wart.

Where HPV prevalence is defined as the presence of viral DNA, Bauer *et al* (1991) found a prevalence of 30-40% in normal cervical specimens of sexually active women. However, more recent studies found approximately 8-27% of normal cervical samples contained HPV DNA (reviewed by Shiffman 1994) which may more accurately reflect the levels of HPV infection in the general population.

Thirty percent of cutaneous warts spontaneously resolve within 6 months, but persistent HPV infection which is resistant to treatment occurs in a minority of immunocompetent individuals. HPV infections are particularly persistent in clinically immunosuppressed renal allograft recipients (RAR) (shown in Figure 1.6) where the prevalence of HPV infection in this group of patients ranged from 42%-55% in different studies (reviewed by Benton *et al* 1992). Patients receiving immunosuppressive therapy for long duration have a greater likelihood of developing warts. For example, a 20% prevalence of viral warts was found in a group of RAR who had a graft survival of less than 5 years but a 77% prevalence in those patients whose graft survival was between 5-22 years (Barr *et al* 1989). In a study of RAR whose mean graft survival time was 4.5 years, the prevalence of cutaneous warts was 26%. However, RAR with high exposure to sunlight had a 58% prevalence of viral warts (Boyle *et al* 1984). This suggests that UV exposure plays a role in promoting the development of HPV infection. Infection with "low risk" cutaneous HPVs is rarely associated with malignant progression. However, HPV infection may be a co-factor which in combination with immunosuppressive therapy and UV exposure, can lead to the transformation of common warts into squamous cell carcinomas (Noel *et al* 1994). Indeed Stark *et al* (1994) detected HPV-1 or 2 in 10% squamous cell carcinomas from RAR.

Epidermodysplasia verruciformis (EV) is a rare autosomal recessive disease which predisposes individuals to infection with HPV types which are not pathogenic in

immunocompetent individuals (reviewed by Majewski *et al* 1995). HPV-5 and 8 are often associated with this disease but many other HPV types have been detected in these lesions. EV associated HPVs share DNA sequence homology and are shown as a separate branch of the phylogenetic tree (Figure 1.4). Multiple squamous cell carcinomas develop in approximately 33% of EV patients, particularly on UV exposed sites. HPV-5 and 8 are present in 90% of these squamous cell carcinomas and are thought to play a role in malignant transformation of host cells. HPV infection which is clinically and histologically typical of EV, is also found in RAR and high UVB exposure in RAR also increases the prevalence of malignant conversion of warts infected with EV and other HPV types.

1.7 Models of HPV infection and propagation

1.7.1 Mouse xenografts

The study of HPV life cycle and virus-host cell interactions have progressed slowly due to lack of culture systems for the propagation of HPV *in vitro*. Kreider *et al* (1985) were the first to demonstrate productive infection of HPV when normal human cervical tissue infected with condylomata acuminata extracts were implanted into the renal capsule of nude mice. HPV-11 DNA was detected in these infected xenografts and they displayed histological features typical of condylomata. Productive replication of HPV-11 was also observed in experiments using neonatal foreskin epithelium xenografted into the renal capsule of nude mice. Virions isolated from these grafts were capable of infecting foreskin epithelium (Kreider *et al* 1987). However, HPV-1 is the only other HPV type which can be propagated in this system (Kreider *et al* 1990).

Successful production of HPV-1 and 11 in xenografts, depends on the initial isolation of virions from clinical samples. HPV-16 and 18 have not been produced in this system since virus particles cannot be isolated from infections with these

HPV types. However, productive infection of HPV-16 has been demonstrated by grafting a low grade human cervical intraepithelial neoplasia derived cell line containing episomal HPV-16 DNA onto prepared granulation beds in nude mice (Sterling *et al* 1990). The grafted cells formed a stratified differentiating epithelium and exhibited features of low grade cervical intraepithelial carcinoma. This experimental system can support the complete HPV life cycle since HPV particles can be found in the terminally differentiated KC. However, it is not known whether the particles produced are infective virions. Nevertheless, the technique has been useful in localising expression of the L1 and E4 proteins within the differentiating epithelium (Sterling *et al* 1993).

1.7.2 Raft Cultures

The organotypic culture system or raft culture is a model experimental system which mimics the stratified epithelium in skin and therefore can allow productive infection of HPV. Epithelial cells are placed on a dermal equivalent such as a synthetic collagen matrix or de-epidermised dermis, and cultured at the air-liquid interface. Initial studies have shown the production of infective virions from explants of HPV-11 infected xenografts cultured on rafts (Dollard *et al* 1992). HPV production was also demonstrated in a carcinoma cell line, containing episomal HPV-31 DNA, when grown on a raft culture and treated with phorbol ester (Meyers *et al* 1992).

1.7.3 Non-productive HPV infection in vitro

Inoculation of KC with HPV *in vitro* does not lead to a productive HPV infection, but may still provide insight into the early stages of HPV infection and DNA replication. Infection of cultured human KC with HPV-1 virions from plantar warts results in transient HPV DNA replication and viral DNA persists in KC for up to 4 months after infection before it is lost (La Porta *et al* 1982). Subsequent studies have shown viral transcripts in HPV-1 infected human transformed KC (Burnett *et al* 1983), primary human KC (Chow *et al* 1987) and bronchial cell lines (Christian *et*

al 1987). Human KC infected with HPV-1 particles contained HPV-1 specific mRNAs which represent a sub-set of those found in plantar warts and had identical splice patterns (Chow *et al* 1987). The E1^E4 transcript is the most abundant viral mRNA in HPV-1 infected KC (Chow *et al* 1987) and is also found in HPV-11 infected foreskin explants up to 4 weeks after infection (Smith *et al* 1993). Passage of KC outgrown from these explants also had E1^E4 transcripts for up to 42 days in culture.

In a study of virus-host interactions, monocytes infected with BPV produced bioactive IL-1 3 days after infection and viral DNA was present in these monocyte cultures up to 28 days after infection (Bonnez *et al* 1991). After infection of KC with HPV-1 viral DNA could be detected up to 13 days, and was not replicated under these conditions (Bossens *et al* 1992). Studies of HPV infection using HPV-11 VLPs, demonstrated that VLPs bound and were taken up into a variety of cells in different mammalian species (Muller *et al* 1995). This process could be partially blocked in the presence of a neutralising antibody or by pre-trypsinisation of host cells. When VLPs linked to a β-galactosidase (β-gal) reporter plasmid were inoculated into HeLa cell cultures, β-gal expression was detected up to 48 hours after infection but only in 1% of cells. When cultures were co-infected with replication-deficient adenovirus and VLP-β-gal, expression of β-gal increased by 10- 20 fold. This indicates that release of VLP-β–gal from lysosomes occurred at low levels without the presence of adenovirus which is known to lyse lysosomal membranes (Muller *et al* 1995)

1.7.4 Transfection in vitro

Much of the knowledge of HPV replication comes from *in vitro* transfection studies. Oncogenic HPVs such as HPV-16, 18 and 33, when transfected into cells, integrate into cellular genomes and cause transformation (Durst *et al* 1987; Gilles *et al* 1993). In contrast non-oncogenic HPVs, such as HPV-11, generally do not integrate but

replicate transiently when transfected into human KC (Mungal *et al* 1992) and squamous cell carcinoma cell lines (Del Vecchio *et al* 1992).

1.8 Immunity to HPV

Persistent HPV infection is observed in patients with clinical immunosuppression due to Hodgkin's disease, human immunodeficiency virus infection, inherited disorders such as Wiskott Aldrich syndrome and therapeutically immunosuppressed RAR (reviewed by Benton *et al* 1992). Patients with diseases consisting of deficiencies in humoral immunity do not show such susceptibility to infection with HPV (Frazer *et al* 1992). This observation suggests CMI plays a more central role in controlling the course, duration and regression of HPV infection. However, rabbits and dogs vaccinated with CRPV and COPV VLPs respectively, are protected against experimental infection with PV (Breitburd *et al* 1995; Suzich *et al* 1995). Passive transfer experiments indicated protection from infection was mediated by L1 specific antibodies. However, the role of humoral responses in HPV infection is less clear.

Since HPV does not productively infect monolayers of cells *in vitro*, conventional virus titre assays and isolations cannot be performed. Thus the identification of infected individuals is not possible using conventional methods. The study of HPV-specific immune responses has proved difficult largely due to a lack of suitable HPV-antigens for use in both antibody and cellular assays, although the production of VLPs has now provided an abundance of antigen to use in serological assays of some HPV types. Since there is no reliable non-invasive specific test to determine past or present HPV infection, information provided by patients has to be relied on for infection history. Indeed normal tissue may harbour HPV DNA (Young *et al* 1989; Ferenczy *et al* 1985) suggesting either latent infection which may later develop into clinical infection, or DNA remaining after an infection has cleared.

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generated which identified four L1 epitopes. Altman *et al* (1992) generated $CD4^+$ T_H cell lines specific for HPV-16 E7 peptides which recognised three HPV-16 E7 epitopes using overlapping peptides.

Cytotoxic T cell (CTL) epitopes of HPV-16 E6, E7 and L1 have been identified in mice (Stauss *et al* 1992) and more recently human CTL specific for HPV-11 E7 peptides were demonstrated which were HLA-A2 restricted (Tarpey *et al* 1994). Interestingly, some $CD4^+$ T_H cell lines generated towards HPV-16 E7 peptides exhibited CTL activity *in vitro* (Altman et al 1992).

Natural killer (NK) cell activity from PBMC has been demonstrated towards KC which contain HPV-16 genomes, and decreased NK lysis was shown in PBMC from patients infected with HPV (Malejczyk *et al* 1989). Other studies could not demonstrate NK mediated lysis of HPV-16 infected KC, although pretreatment of freshly isolated PBMC with IL-2 generated lysis of these targets, which increased when target cells were pretreated with γ-IFN (Wu *et al* 1994).

1.8.2 Local cell mediated immunity

The study of cells within warts which play a role in skin immunity provide insight into the way in which HPV infection alters local immunity. Immunohistochemical studies of normal human epidermis have detected low numbers of T cells, with CD8 : CD4 T cell ratios of 3:2 (Foster *et al* 1990), while normal human mucosal epithelium had equal ratios of these T cell subsets (Tay *et al* 1987). No predominance of either T cell subset was found in the dermis of cutaneous or mucosal warts using semi-quantitative analysis (Viac *et al* 1992a; Viac *et al* 1992b; Chardonnet *et al* 1986; Tay *et al* 1987). Other studies found either a predominance of $CD4^+$ (Bishop *et al* 1990) or $CD8^+$ T cells (Viac *et al* 1992b) in the stroma of anal and laryngeal warts, while a predominance of $CD8^+$ T cells were found in the epithelium of cervical warts (Tay *et al* 1987). During regression of plane warts there

is an increase in the numbers of T cells infiltrating the epidermis, with a predominance of $CD8^+$ cells (Iwatsuki *et al* 1986), thus suggesting a role for $CD8^+$ T cells in wart regression. However, other studies observed a mononuclear cell infiltrate in the epidermis of regressing plane and genital warts comprising monocytes and equal numbers of $CD8^+$: $CD4^+$ T cells (Aiba *et al* 1986; Fierlbeck *et al* 1989). NK cells were not observed in either regressing plane (Aiba *et al* 1986) or genital warts (Fierlbeck *et al* 1989), suggesting that NK cells do not have an important role in the regression of warts.

In an animal model of HPV infection, syngeneic HPV-16 E7 expressing KC are grafted onto mice therefore presenting HPV-16 E7 to the immune system in a similar way to natural HPV infection (McLean *et al* 1993). Challenge of the grafted mice with HPV-16 E7 vaccinia recombinants results in a delayed type hypersensitivity response (DTH) which was mediated by $CD4^+$ T cells (McLean *et al* 1993). Immunohistochemical analysis of ears from grafted mice subsequently challenged with vaccinia E7 recombinants showed infiltration of predominantly activated $CD4^+$ T cells and macrophages (Chambers *et al* 1994). A threshold dose of grafted sensitising cells was observed below which a DTH response could not be induced upon challenge. When a subthreshold dose of E7 expressing cells were grafted, an unresponsive state was induced and mice could not be further sensitised with high doses of E7 to induce a DTH response (Chambers *et al* 1994). This suggests that low levels of HPV antigen presented to the immune system in a natural infection may cause tolerance or an anergic state representing an evasion strategy by the virus and may explain the persistent nature of HPV infections. Chen *et al* (1991) implicated $CD8^+$ T cells in the rejection of HPV-16 E7 expressing tumour cells in mice vaccinated with HPV-16 E7 expressing cells.

Another possible strategy for HPV to evade the immune system may be effected through the reduced numbers of LC observed in HPV infection. Whether this

reduction is a cause or consequence of HPV infection is not known. Further discussion of LC is found in Chapter 3. HPV may also alter local immune responses by modulating ICAM-1, VCAM-1 and E-selectin expression on the surface of vascular endothelial cells which regulate the entry of leucocytes into the skin (discussed further in chapter 3). The presence of activated leucocytes within the skin contribute to the local cytokine production by LC, KC, melanocytes, dendritic cells, fibroblasts and mast cells, and alterations of this cytokine network can determine the outcome of a cutaneous infection. Acute viral infections often stimulate a T_H1 cytokine response thus promoting CMI whereas chronic infections fail to stimulate this response, but generate a T_H2 mediated response. This immune reaction drives a humoral response which may not be appropriate for clearing a chronic intracellular infection (Sher *et al* 1992). HPV often causes a chronic infection of the epithelia but the cytokines produced by the host in response to this infection are unknown. The effect of HPV on cutaneous cytokine responses are discussed further in chapter 4.

1.9 Aims

The aims of the following study were to:

1) Generate HPV-specific T_H cell clones and to further characterise the specificity and function of these cells.

2) Quantify numbers of $CD3^+$ and $CD45RO^+$ T cells in the dermis and epidermis of cutaneous warts. Determine the density of LC in the epidermis, and ICAM-1 and E-selectin expression, in the dermis of cutaneous warts.

3) Undertake an adherence assay of activated T cells to sections of cutaneous warts and compare this adhesion with that seen in normal skin and inflammatory skin disease.

4) Determine the cytokine mRNA profile in cutaneous warts and compare this with cytokine expression in normal skin.

5) Establish an *in vitro* system to model the early stages of HPV infection, and determine whether HPV directly modulates cytokine expression in KC.

Chapter 2
T cell proliferation to purified HPV

2.1 Aim

To evaluate the role of cellular immunity in HPV infection, T_H proliferative responses to purified HPV antigen were investigated *in vitro*. In addition the generation of T cell clones to HPV was attempted in order to determine the role of CMI *in vivo* .

2.2 Introduction

The immunology of cutaneous warts is not fully understood and it is uncertain what factors lead to clinical infection, affect the duration of the disease, and trigger wart regression. Evidence for CMI playing a greater role in immunity towards HPV than humoral responses arise from the observations that RAR have an increased incidence of cutaneous warts (Rudlinger *et al* 1986; Barr *et al* 1989). Patients with a defect in the humoral arm of the immune system do not show a similar increase (Frazer *et al* 1992). T cells are key regulators of CMI and the *in vitro* proliferative response of TH cells to specific antigen is thought to reflect both the memory and the size of the systemic CMI response. T cell proliferative responses to purified HPV *in vitro* was either weak or absent in individuals with either progressing or regressing cutaneous warts (Charleson *et al* 1992). Clonal T lymphocytes may be generated from PBMC which show an HPV specific response *in vitro*. Indeed, by isolating and expanding a clone of T cells, any negative influence which could affect its proliferative capacity in culture would be removed and increased proliferation may result.

The generation of HPV-specific clones of T cells *in vitro* can provide information

about which antigen specific lymphocytes may be involved in immune responses towards HPV. This may further the understanding of the pathogenesis of this virus and aid the design of effective vaccines and therapies. The specificity and function of T cell clones generated from patients infected with HPV is a part of the immune response which can be dissected and studied *in vitro*. The MHC restriction of T cell clones may also be examined therefore identifying HLA haplotypes which can potentially restrict HPV-specific T_H responses.

As discussed in chapter 1 it is difficult to obtain an accurate clinical history of a patient with regard to cutaneous HPV infection. Eighty five percent of individuals without current cutaneous warts have HPV-1 specific serum antibodies (Steele *et al* 1990), indicating previous exposure to HPV-1. However, antibody response is not correlated with lymphoproliferation to purified HPV-1 or 2. In addition, another study found no correlation with either the HPV-specific antibody response or CMI and the presence of koilocytes (Cubie *et al* 1988). Therefore in this study, PBMC from individuals either with or without current HPV infection were screened for lymphoproliferative responses to the virus *in vitro* before proceeding with cloning.

The strategy taken to obtain HPV-specific T cell clones was to culture PBMC together with purified HPV and then expand the numbers of T cells from this initial stimulation. Cultures were then screened for responses to HPV and positive cultures were cloned by limiting dilution. Alternatively, PBMC were stimulated initially with HPV and then cloned directly from this cell population without additional expansion.

Currently there are no *in vitro* culture systems able to produce HPV particles in sufficient quantity to use as antigen for lymphoproliferation assays or T cell cloning, therefore, HPV was purified from clinical samples. Autologous APC were also not available in large numbers. The number of responding T cells were increased by

stimulation with anti-CD3 antibody coated tissue culture plates. This procedure has been reported to maintain the antigen specificity of the response (Garbrecht *et al* 1988). In general, lymphoproliferative responses to HPV *in vitro* are low (Cubie *et al* 1988), possibly due to a suppressive effect of HPV on PBMC (Chopra *et al* 1991). In a previous study, *M.leprae* specific T cell clones could only be generated using allogeneic APC (Modlin *et al* 1986). Therefore the absence of autologous APC during culture on antibody coated plates may be advantageous. The strategy of increasing cell numbers non-specifically before cloning and the use of anti-CD3 antibody coated plates in the maintenance and expansion of cell lines has been described previously (Ferrarini *et al* 1990; Yanelli *et al* 1990).

Immunity to viral infections requires CMI, namely $CD8^+$ MHC class I restricted CTL. However, it is the $CD4^+$ T_H cell which is an important initiator of this response and provides help to CTL. Therefore expansion of the $CD4^+$ T cell subset is required to generate a T_H clone. Previous studies, where anti-CD3 antibody was used to stimulate T cells *in vitro* for long periods, showed a gradual decrease in the antigen specific response, and an enrichment of $CD8^+$ T cells (Crossland *et al* 1991). However, signalling via the TCR alone is not sufficient for optimal interleukin-2 (IL-2) production, and proliferation of $CD4^+$ T cells. Furthermore, stimulation of the CD3-TCR complex in the absence of co-stimulation (usually provided by APC) can lead to an anergic state (Allison, 1994). Indeed, stimulation using only anti-CD3 antibody coated plates, induced the death of T_H1 clones (Liu *et al* 1991). Therefore, additional antibodies were coated on plates together with anti-CD3 to avoid these problems.

CD28 is a major co-stimulatory molecule on T cells which prevents clonal anergy when bound by its natural B7 ligands present on APC (Allison, 1994). Clonal anergy is also prevented when anti-CD28 antibodies are added in solution to cultures of T cells stimulated by anti-CD3 antibodies (Harding *et al* 1992). Furthermore,

Riddell *et al* (1990) used soluble anti-CD3 antibody and anti-CD28 antibody, with APC to cross link the antibodies, as a strategy for cloning and expanding human T cells *in vitro*.

Anti-CD4 antibodies have also been shown to synergise with anti-CD3 to induce proliferation of $CD4^+$ T cells when immobilised on tissue culture plates (Emmerich *et al* 1987). Therefore, it was investigated whether the addition of anti-CD28 or anti-CD4 to anti-CD3 coated tissue culture plates generated proliferation of $CD4^+$ T cells *in vitro*. The optimum coating procedure was then used to expand T cell numbers before cloning.

2.3 Materials and methods

2.3.1 Isolation of peripheral blood mononuclear cells

Ethical permission for this study was granted following review by a local ethics committee, and was carried out in accordance with standards laid down in the 1964 declaration of Helsinki. Following informed consent, heparinised blood from patients with current HPV infection attending the Department of Dermatology, Royal Infirmary of Edinburgh or from normal healthy volunteers with no evidence of current HPV infection, was separated by centrifugation of undiluted blood over an equal volume of lymphoprep ™ (Nycomed UK, Sheldon, Birmingham, UK) at 800 *g* for 30 minutes at room temperature. The interface containing the PBMC was removed and washed three times in sterile phosphate buffered saline (PBS). The isolated PBMC were resuspended in RPMI-1640 (Gibco-BRL, Paisley, Scotland, UK) containing 10% autologous plasma, 100 IU / ml penicillin, 100 µg / ml streptomycin (Sigma, Poole, UK), and 2 mM L-glutamine (Gibco) (autologous GM).

2.3.2 Purification of HPV

HPV was purified using a modification of a previously described method (Gissmann

et al 1976). Briefly, tissue parings from cutaneous warts were removed as part of normal treatment and stored in PBS with 100 IU / ml penicillin and 100 µg / ml streptomycin at -20°C. Parings from 20 individuals were cut into small pieces and homogenised in a small volume blender. The supernatant was aspirated and kept on ice after gross debris was allowed to settle. The remaining pieces from the blender were ground up in a mortar and pestle with sea sand, removing the supernatant during the process, until no large pieces remained. The supernatant was centrifuged at 1000 *g* for 15 min to remove any gross debris followed by ultra-centrifugation at 80,000 *g* for 75 min at 4°C to pellet virus. The pellet containing virus was then resuspended in 40% caesium chloride in PBS (refractive index 1.367) and adjusted to a refractive index of 1.365 with either PBS or caesium chloride. This virus suspension was then centrifuged at 110,000 *g* for 20-24 hrs at 18°C. The opaque band of virus particles was visualised under an inverted light microscope and the band corresponding to 1.34 g / ml density containing the virus was aspirated using a glass pasteur. CsCl was removed by dialysis against PBS overnight at 4°C, followed by inactivation with 1000 J / m^2 UVB radiation for further handling. The preparation was then filter sterilised by passing the preparation through a 0.2 µm filter. The presence of virus was confirmed in each preparation by negative staining with 2% phosphotungstic acid for 2 min and visualised by transmission electron microscopy. The quantity of protein in each virus preparation was estimated by Biorad assay according to the manufacturers instructions (Biorad, Hemel Hempstead, UK) which was performed on individual batches of HPV. However, many batches of virus were below the detection limit of this assay, therefore titration in lymphoproliferation assays was the preferred method of quantification. A limited number of batches were analysed by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Silver staining (Biorad), and in some cases Western blotting onto nitrocellulose membranes (Amersham, Bucks, UK). These were then probed with a monoclonal antibody to HPV-1 L1 (Seralab, Crawley Down, UK). Primary antibody was detected with a

rabbit anti-mouse horseradish peroxidase conjugated antibody (DAKO, High Wycombe, UK) an developed with diaminobenzidine (Sigma) as described in chapter 3.

2.3.3 Lymphoproliferative responses to HPV particles

Triplicate 200 µl cultures were established with 2 x 10^5 PBMC per well with autologous GM in 96 well U-bottomed plates (Nunc, Roskylde, Denmark). Cultures with various concentrations of HPV particles or controls without antigen were incubated at 37°C in 95% air / 5% CO_2 for seven days. Wells were pulsed with 0.75 µCi ^3H methyl thymidine (Amersham) on day 6 of culture and harvested 16 - 24 hrs later. Cells were harvested onto glass fibre filters using an Automash 2000 cell harvester (Dynatech, UK), and the activity incorporated was measured by liquid scintillation counting. One ml hydro luma scintillant (Lumac, Belgium) was dispensed per vial before counting on a 1900CA tri carb liquid scintillation analyser (Canberra Packard, UK). Proliferation is shown either as counts per minute (cpm) or as stimulation index (SI) which was calculated as the cpm of cultures with HPV divided by the cpm of cultures with medium only.

2.3.4 Initial stimulation of cultures

Five to ten cultures were established in wells of a 96 U-bottomed plate in parallel to the thymidine incorporation assay for a primary *in vitro* stimulation with purified HPV as antigen. Recombinant human IL-2 (20 IU / ml), (Genzyme, Cambridge, MA, USA) was added on days 5 and 8 of culture before harvesting on day 9. Cell viabilities, as determined by trypan blue exclusion, were always > 80% and frequently ≥ 95%. Harvested cells were cultured at 1 x 10^6 / well of 24 well plates coated with the following antibodies: 1) anti-CD3, 2) anti-CD3 and anti-CD4 and 3) anti-CD3 and anti-CD28. Cells were cultured in RPMI-1640 supplemented with the same additives as autologous GM except 10% heat inactivated pooled human plasma was added (pooled human GM). Twenty IU / ml IL-2 was also added to this GM.

After expansion of T cells for 1-4 weeks, the proliferative response to HPV particles was assessed as outlined in section 2.3.3. When proliferative responses were observed, these cell lines were cloned by limiting dilution. In some experiments cloning was performed directly after the primary stimulation with HPV.

2.3.5 Coating plates with monoclonal antibodies

Flat bottomed 96 well plates (Falcon, USA) were coated with 50 µl of a 10 µg / ml goat anti-mouse polyclonal antibody (Sigma) by incubation overnight in Tris buffer pH 9.4 at 4°C. Plates were washed 3 X with 200 µl PBS / well before coating with the monoclonal antibodies in 1% bovine serum albumin (BSA) in PBS for 2 hrs at 37°C. Sequential coating of plates was performed with 50 µl mouse anti-human CD3, and monoclonal antibodies to either CD4 or CD28 (DAKO) . Finally unbound antibody was removed by washing the plates 3 X in PBS. Plates were stored at 4°C for up to 1 week. To determine the optimum concentration for coating, the antibodies were titrated in a checker board style, and the proliferation of PBMC measured after 4 days.

2.3.6 Expansion and maintenance of cells on coated plates

Cells from the primary stimulation were cultured in pooled human GM containing 20 IU / ml IL-2 at 1 x 10^6 / ml in each well of a 24 well antibody coated plate. Cells were transferred to fresh wells before they became overcrowded and transferred weekly onto new plates, with fresh GM and IL-2 every 3 days. The phenotype of the enriched cell population was determined by immunofluorescent staining as follows: Mouse anti-human monoclonal antibodies to CD4, CD8 and CD25 were incubated with 1 x 10^6 cells (harvested from coated tissue culture plates at various time points) for 1 hr at 4°C, washed once in 1% BSA / PBS containing 0.01% sodium azide (wash solution) and incubated for 60 min with fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse IgG (Sigma) before washing twice in the wash solution. Flow cytometric analysis was performed using a Coulter XL (Coulter, Hiyaleah, FL, USA) and the data analysed using XL 1.5 software.

2.3.7 Screening cultured cells for lymphoproliferative responses to HPV particles

Cells cultured in antibody coated wells were harvested at least 6 days after the last stimulation and washed once in PBS and the viability determined as before. Cells were cultured in 96 well U-bottomed plates with various numbers of gamma-irradiated PBMC and purified HPV antigen. Cultures were incubated at 37 ^{o}C in 5% CO_2. In general, 5 x 10^4 T cells and 1 x 10^5 APC / well were used. Cultures were harvested after 72 hr, although other times were also studied. APC were either freshly isolated PBMC or recovered from frozen stocks kept under liquid nitrogen. Proliferation of either autologous or allogeneic APC was inhibited by γ-irradiation (5000 RADS).

2.3.8 T cell cloning

Cells were harvested from primary cultures stimulated with purified HPV or from secondary or tertiary stimulations on antibody coated tissue culture plates. In some experiments T cell blasts were purified by separation on either Lymphoprep™ or a discontinuous percoll (Sigma) gradient. Frozen autologous PBMC were recovered into autologous GM, washed once and irradiated (5000 RADS) before use as APC. T cell blasts were plated out at 30, 3 and 0.3 cells per well in 20 µl autologous GM into Terazaki wells (Sterilin, UK) with 1 x 10^4 APC, 20 -200 U / ml IL-2 and purified HPV at the optimum concentration. Between 60 - 300 wells were plated out as the cell yield permitted. Alternatively, PBMC were stimulated for 7 days before harvesting on lymphoprep™. These cells were then plated out in 96 well U-bottomed plates at 10 T cell blasts per well with 1 x 10^5 autologous APC. Cultures contained IL-2 (20 IU / ml) and HPV particles in 200 µl autologous GM. Plates were screened by light microscopy for proliferating cultures for up to 14 days after initiation. Any cultures containing proliferating cells were transferred to a fresh well of a 96 well U-bottomed plate with autologous GM containing IL-2, autologous APC and antigen.

2.4 Results

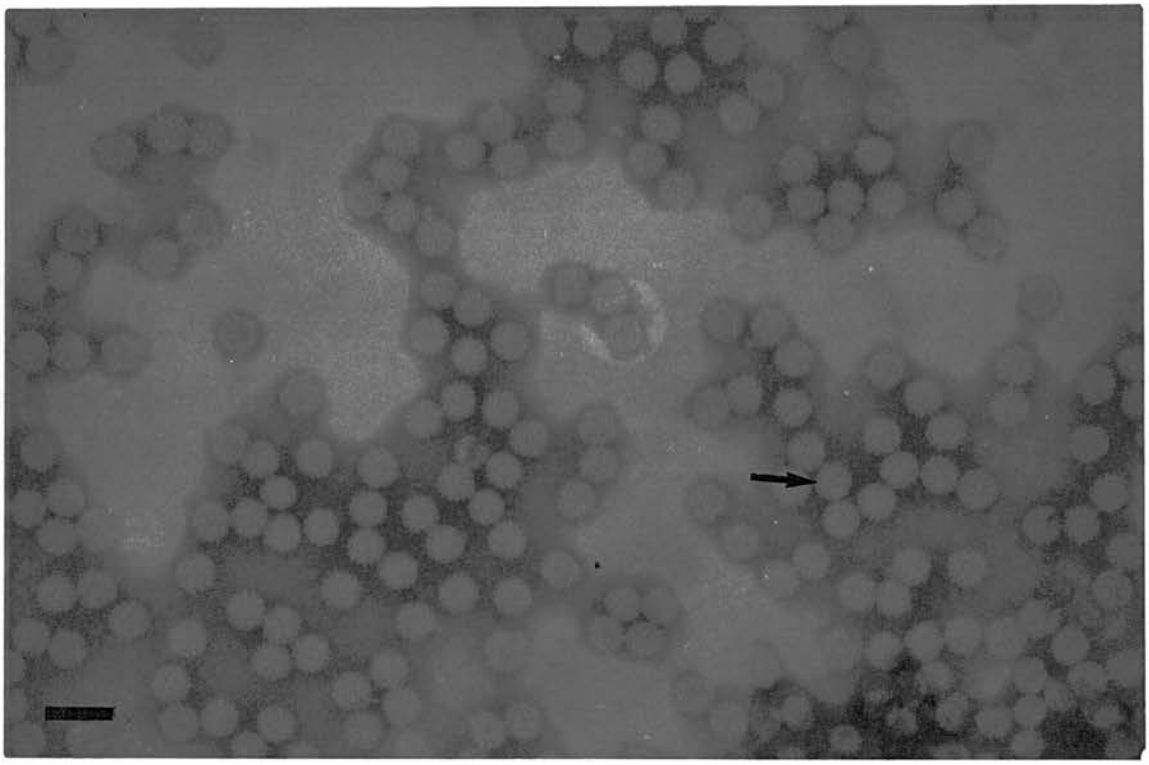
2.4.1 Antigen preparation

Since large quantities of HPV cannot be obtained *in vitro* virus particles were purified from patients' tissue parings, as described in section 2.3.2. HPV do not productively infect monolayer cultures of KC *in vitro* . Therefore it was not possible to obtain an accurate virus titre for each batch of purified HPV. Four methods were used to characterise and standardise the different preparations of virus. Firstly, in order to confirm the presence of virus, transmission electron microscopy was performed after negative staining with 2% phosphotungstic acid. Figure 2.1 shows an electron micrograph of one batch of virus particles at a magnification of 75000 x. This was the only effective method of determining if HPV was present and it is estimated that 1 x 10^8 particles / ml are required to allow visualisation.

In the second method total protein was quantified using the Biorad assay. This method was only useful for preparations with a high titre of virus. Many batches of virus were below the sensitivity of the assay, although other batches contained as much as 100 μg / ml. The three batches (1, 2 and 4) described here contained 80, 25 and < 7 μg / ml.

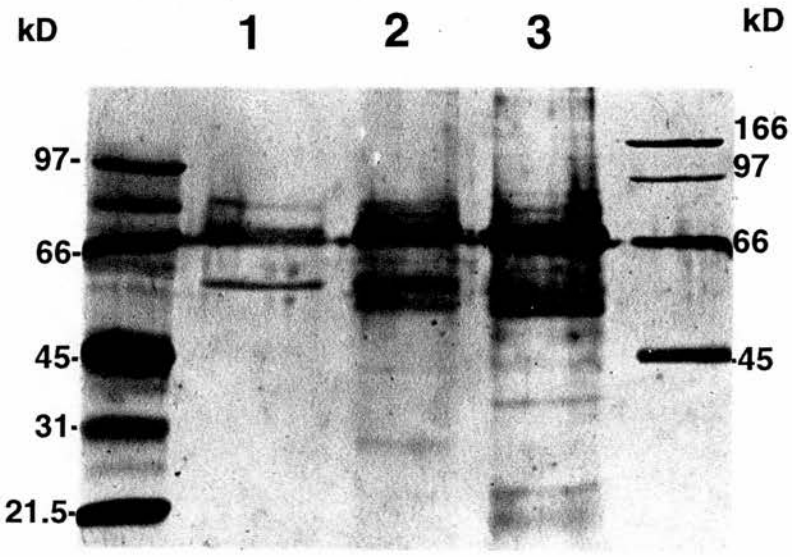
The third method of characterising batches of virus was by separation on SDS-PAGE followed by silverstaining, or Western blotting with HPV-1 L1 specific antibody. The results obtained with batches 1, 2 and 4 are shown in Figures 2.2 and 2.3.

Figure 2.1 Electronmicrograph of purified HPV (75000 X)



Bar = 100 nm. All HPV were complete with visible capsomeres (as indicated). No empty particles were observed.

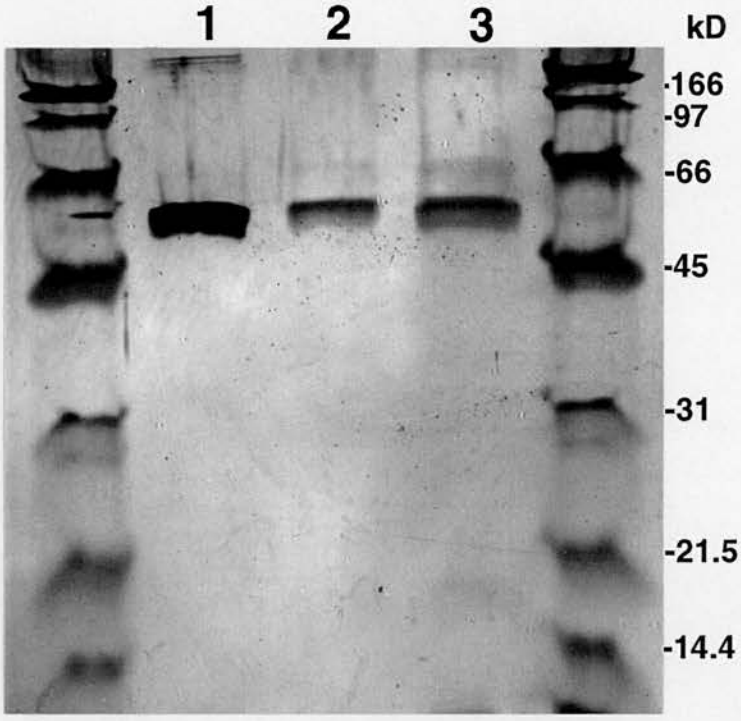
Figure 2.2 SDS-PAGE of purified HPV



Each batch of purified HPV was boiled for 5 min with 5 % 2-mercaptoethanol and 2% SDS and resolved by 8% SDS-PAGE followed by silver staining. Lanes 1, 2 and 3 show 0.45 µg batch 2, 0.75 µg batch 1 and 2.4 µg batch 4 of purified HPV respectively. Batches 1, 2 and 4 were derived from tissue parings which were clinically diagnosed as HPV-2, HPV-1 or undiagnosed cutaneous viral warts respectively.

The main structural proteins of HPV are L1, the major capsid protein and L2 , the minor capsid protein. L1 has a molecular weight of 55 kD and L2 is 76 kD in size (Pfister *et al* 1994). Both structural proteins can be observed as major bands on the gel (Figure 2.2). However, the predominant protein in the three preparations has a molecular weight of 66 kD. This band could either be another species of L2, or a degradation product of L2 which is 65 kD (Doorbar *et al* 1987), or contaminating keratins from host cells.

Figure 2.3 Western blot of three different purified HPV batches

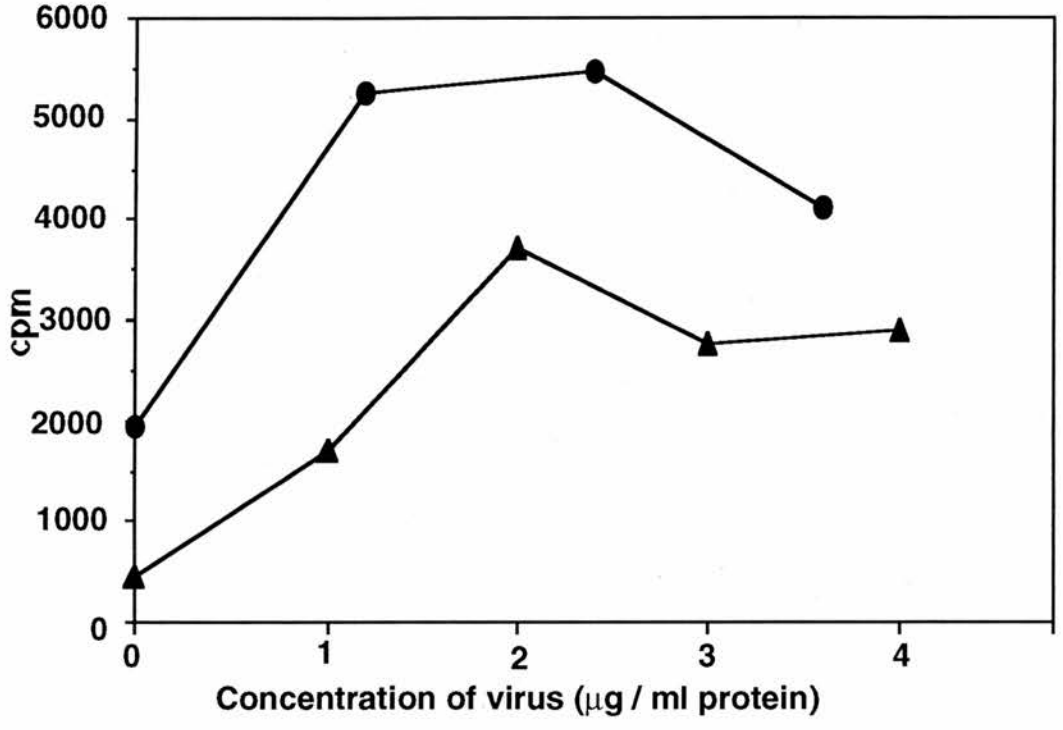


Batches 1 (0.75 μg), 2 (0.45 μg), and 4 (2.4 μg) of purified HPV were separated by SDS-PAGE, blotted onto nitrocellulose and probed with a monoclonal mouse anti-HPV-1 L1 primary antibody as described in section 2.3.2. Lanes 1, 2 and 3 show batches 2, 1 and 4 (respectively).

The 55 kD HPV-1 L1 protein can be clearly identified in all 3 HPV batches in Figure 2.3. Although Batch 2 (Lane 1) had the least amount of protein loaded on the gel it had the greatest abundance of HPV-1 L1. This indicated that most of the pairings used to generate this batch were infected with HPV-1. Lane 2 (batch 4) contained HPV purified from wart pairings diagnosed as HPV-2. This indicates contamination of HPV-1 in this batch. Batch 1 (Lane 3) contained HPV-1 as expected.

A fourth method of standardising preparations of HPV was to titrate the amount of virus used to generate optimum proliferation *in vitro*. Figure 2.4 shows the proliferation generated from PBMC of 2 different donors to 2 different HPV batches.

Figure 2.4 Determination of the optimal amount of purified HPV for lymphoproliferation *in vitro*



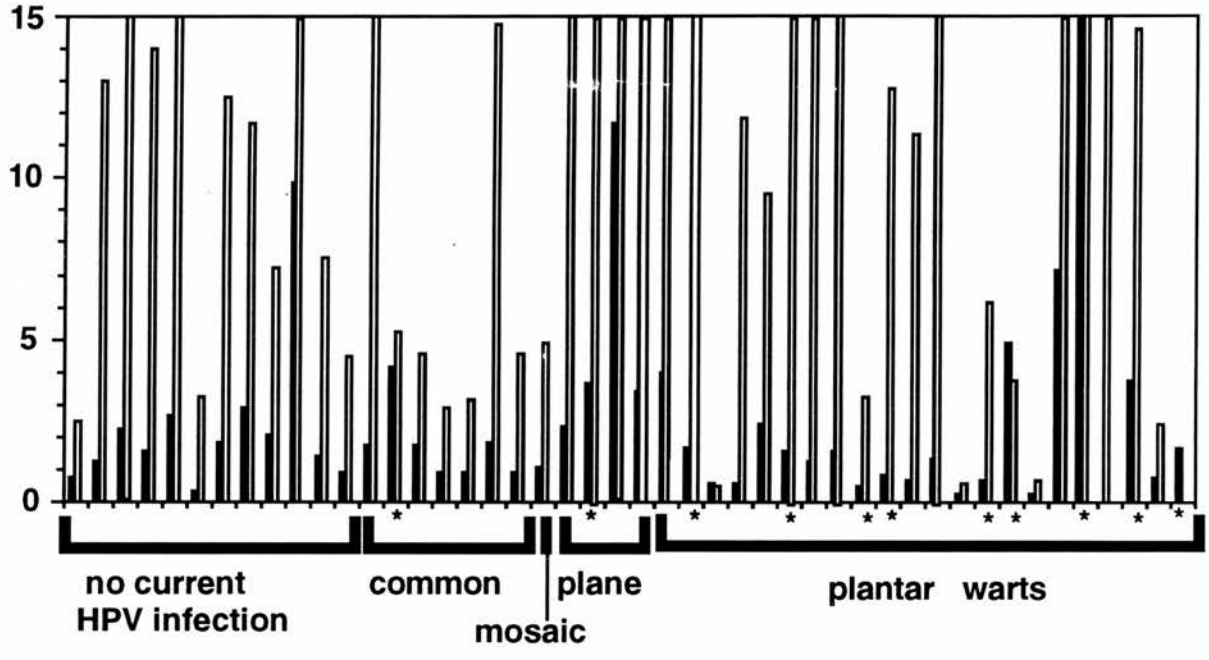
● = Donor 15 (stimulated with batch 4), ▲ = Donor 5 (stimulated with batch 1). standard error of the mean (SEM) of triplicates was calculated and was < 14% mean. The concentration of purified HPV producing optimal proliferation of PBMC was determined by a 7 day thymidine incorporation assay described in section 2.3.3.

Increasing the protein concentration of virus in the culture increased proliferation, with the maximum at a concentration of 2 µg / ml. This was repeated 6 times, and all individuals with or without HPV infection produced an optimum proliferation in the range of 1 - 2.5 µg / ml. Two µg / ml of batches 1 and 4 was used in all subsequent assays to screen other PBMC or for cloning.

2.4.2 Screening patients

In order to generate T cell clones from either asymptomatic individuals or patients with current HPV infection, lymphoproliferative responses from each donor were assessed to determine which donors had previous HPV exposure and could mount T cell responses *in vitro*. PBMC from each individual selected was screened as described in section 2.3.3

Figure 2.5 Lymphproliferative responses to purified HPV



PBMC were tested from the following groups of patients : no current HPV infection, common warts, plane warts, plantar warts and mosaic warts. ■ = HPV , □ = PHA (1 μg / ml). * = warts which were regressing at the time blood was taken. Results are shown as stimulation indices (SI). Proliferation of PBMC (medium only) = 3656 cpm ± 535 SEM.

Proliferative responses obtained were variable within each patient group and no marked difference in amplitude of responses was observed from PBMC of patients with current cutaneous warts or individuals without current HPV infection. An SI > 3 was taken as a positive response, and by this criteria 29% (10/34) of all patients with current HPV infection showed a lymphoproliferative response to HPV. In contrast only 8% (1/12) of individuals without current HPV infection responded to HPV although this difference was not significant (Fishers exact test). When patients were grouped by wart type as shown in Figure 2.5, 75% (3/4) plane, 27% (6/22)

plantar, 14% (1/7) common and 0/1 mosaic warts responded with an SI > 3. A significantly greater proportion of patients with plane warts responded to purified HPV than individuals without current HPV infection ($p = 0.02$). The responses of these four individuals to the mitogen PHA were significantly greater ($p < 0.05$) than the PHA responses from either normal individuals or the other patient groups (data not shown). For further analysis individuals were also divided into groups of subjects whose warts showed no sign of regression and subjects whose warts were regressing at the time blood was taken (including individuals whose warts had resolved). Positive lymphoproliferative responses were observed in 20% (5/24) patients who had non-regressing warts. Forty percent (4/10) patients whose warts were resolving or had resolved showed proliferative responses towards HPV. This difference in proliferative response was not significantly different between these 2 groups, although the patient who gave the highest SI (36) had plantar mosaic warts which had completely resolved within 1 month of the assay.

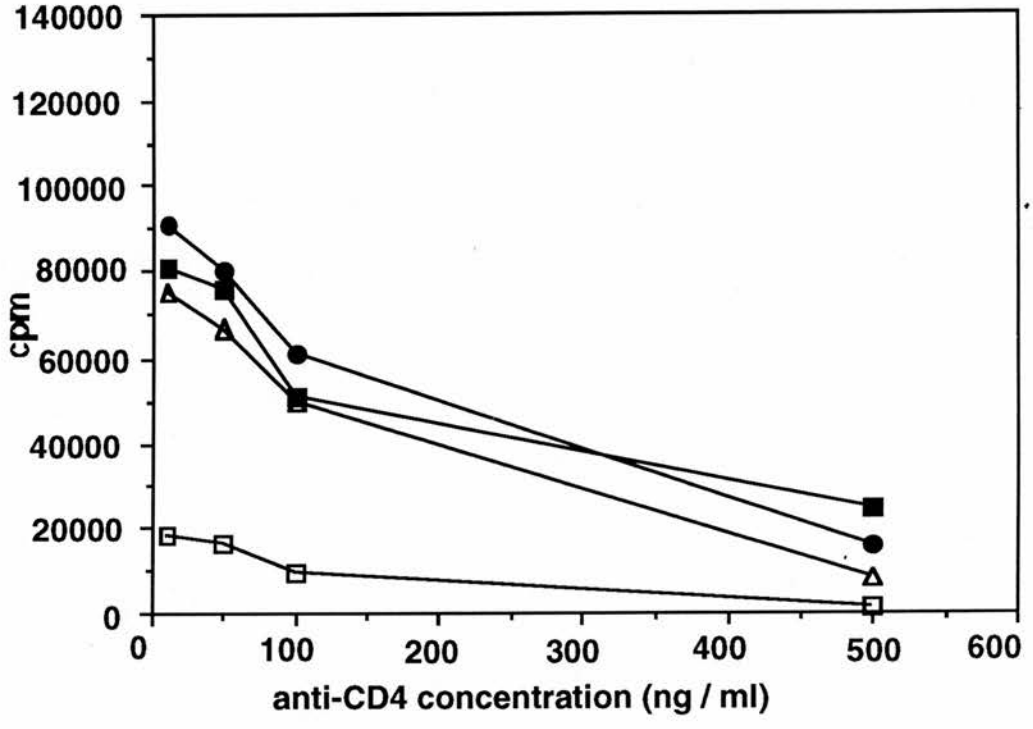
2.4.3 Maintenance and expansion of cell lines

PBMC from donors which generated proliferative responses were then either cloned directly from freshly isolated PBMC, or stimulated initially with antigen and maintained on antibody coated plates. Firstly, it was determined which antibody combination and concentration were optimal. Figures 2.6, 2.7 and 2.8 illustrate the results obtained in proliferation assays with these plates coated with anti-CD3 in combination with either anti-CD4 or anti-CD28.

2.4.4 Titration of anti-CD4 antibody

In order to maintain cell lines on antibody coated tissue culture plates, it was investigated whether a combination of anti-CD3 and anti-CD4 antibodies immobilised on tissue culture plates induced greater proliferation than anti-CD3 antibody alone.

Figure 2.6 Determination of optimal anti-CD3 and anti-CD4 antibody concentrations



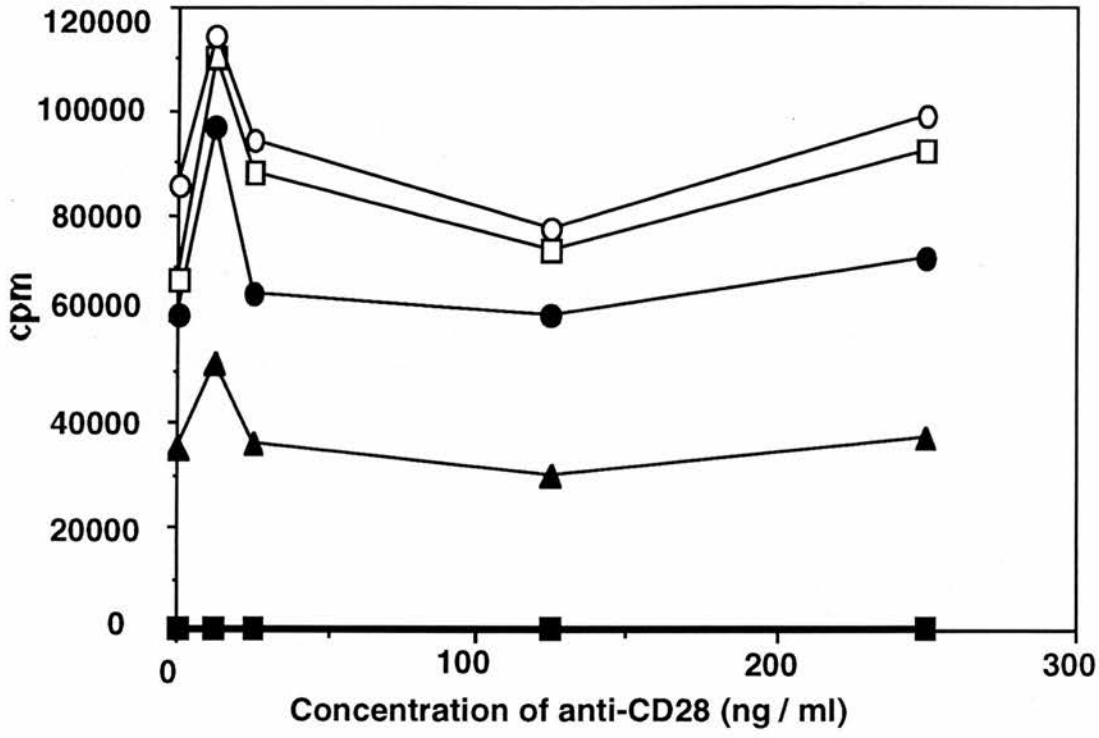
□ = 10, △ = 50, ● = 100, ■ = 500 (ng / ml of anti-CD3 antibody). PBMC previously cultured with soluble anti-CD3 (50 ng / ml) for 5 days were plated out in flat bottomed plates coated with dilutions of anti-CD3 and anti-CD4. 1 x 10^5 cells were plated out per 200 µl well in pooled human GM and a four day thymidine incorporation assay was performed, the results of which are shown above. Responses were: medium = 7,620 cpm; anti-CD3 (500 ng /ml) = 12,9281cpm; anti-CD4 (500 ng / ml) = 554 cpm. SEM ≤ 30% mean of triplicates.

The anti-CD4 antibody when used alone had an inhibitory effect on proliferation compared with the medium only control. Inhibition of proliferation was also observed using the lowest concentration of anti-CD3 antibody (10 ng / ml) with the highest concentration of anti-CD4 antibody (500 ng / ml). All other anti-CD4 antibody concentration combinations had an inhibitory effect on the proliferation to anti-CD3 (500 ng / ml) alone. Increasing anti-CD4 antibody concentrations decreased proliferation. Since the anti-CD4 antibody inhibited the proliferative response of PBMC to anti-CD3 antibody, anti-CD4 antibody was not used in conjunction with anti-CD3 antibody to expand T cell lines.

2.4.5 Titration of anti-CD28 antibody.

It was investigated whether antibodies to the co-stimulatory molecule CD28, when used in conjunction with anti-CD3 antibody increased the proliferative response compared with proliferation induced by anti-CD3 antibody alone (Figure 2.7).

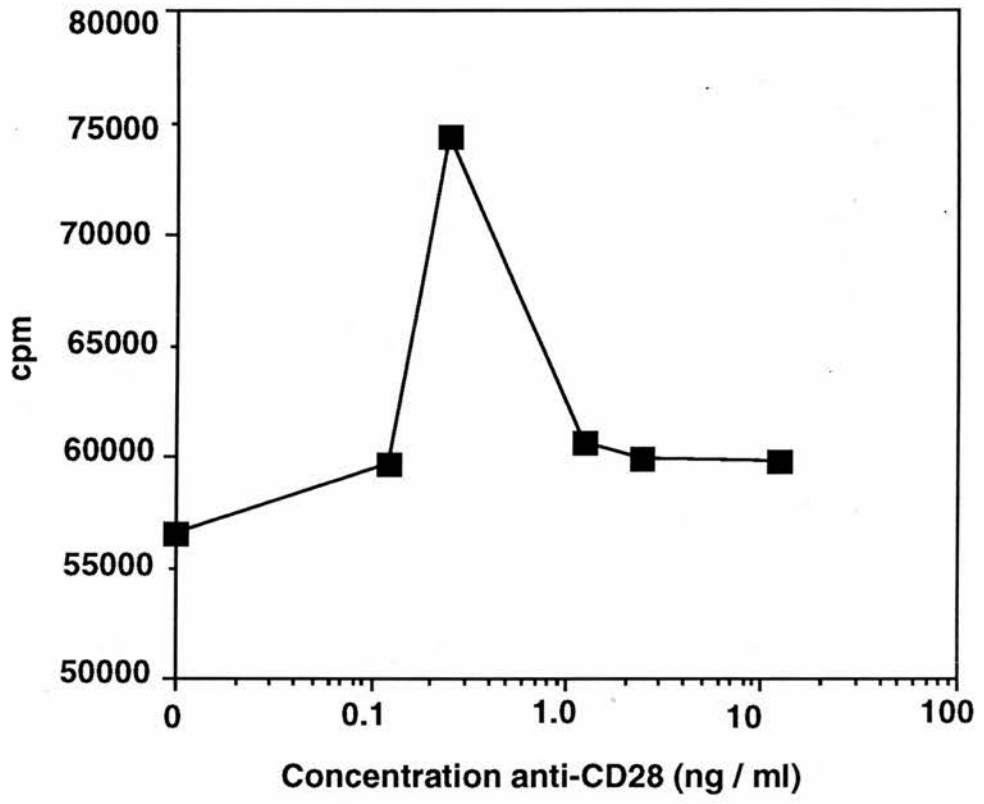
Figure 2.7 Determination of optimal anti-CD28 and anti-CD3 antibody concentration



■ = 0, ▲ = 50, ● = 100, □ = 500 and O = 1000 ng / ml anti-CD3. PBMC previously stimulated with 50 ng / ml anti- CD3 were plated out at 1 x 10^5 cells / well in 96 well plates. Triplicate cultures in 200 µl pooled GM were established for a 4 day thymidine incorporation assay. Figure 2.7 shows the cpm obtained from PBMC exposed to various concentrations of anti-CD28 on plates coated with 0 -250 ng / ml anti-CD3. SEM < 11% mean of triplicates.

The addition of anti-CD28 antibody to the anti-CD3 antibody coated plates resulted in enhanced proliferation compared with plates coated with anti-CD3 alone. A dose of 12.5 ng / ml anti-CD28 antibody gave the greatest proliferation, at all anti-CD3 antibody concentrations. However, the end point had not been reached since anti-CD28 antibody gave greater proliferation and the results are shown in Figure 2.8.

Figure 2.8 Further determination of optimal anti-CD3 and anti-CD28 antibody concentrations



Anti-CD28 antibody was further diluted in combination with a sub maximal dose of anti-CD3 antibody (100 ng / ml). The same assay conditions were used as in Figure 2.7. SEM < 17% mean of triplicates.

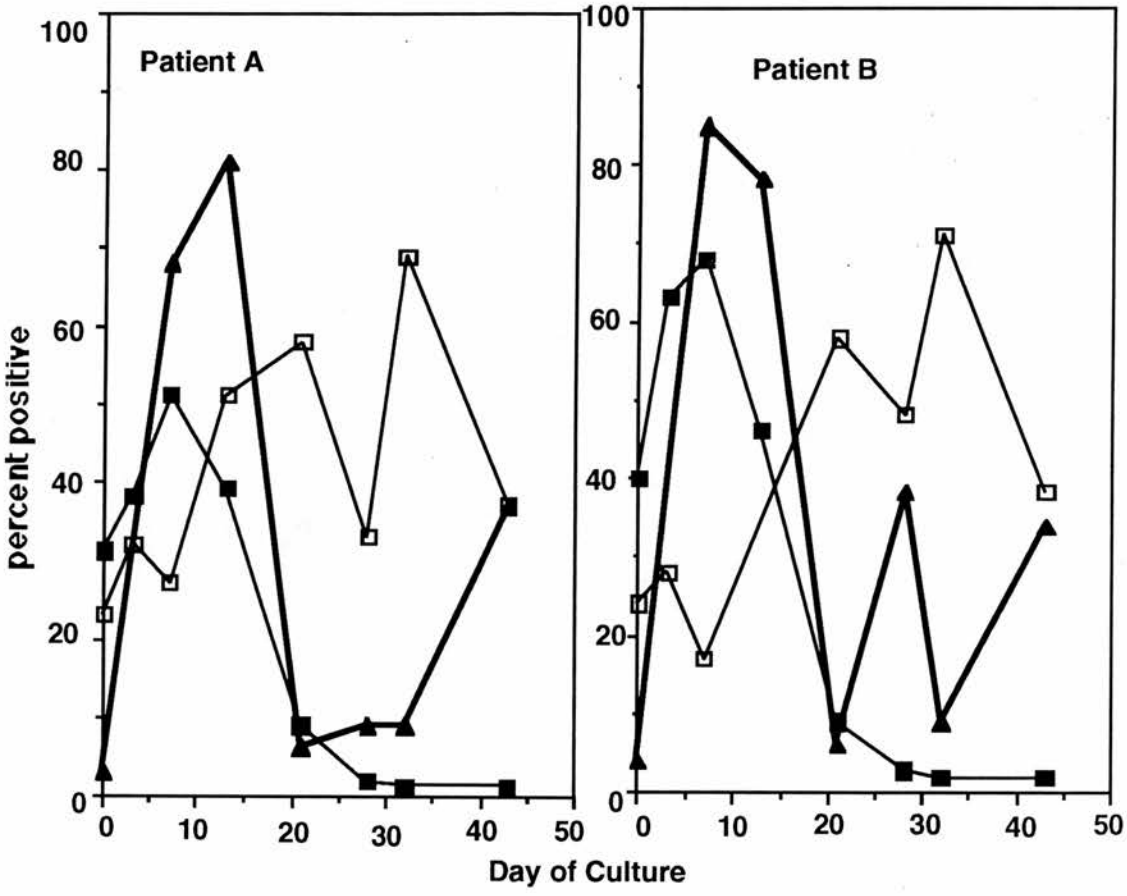
Figure 2.8 shows that optimum proliferation was achieved with 0.25 ng / ml anti-CD28 antibody in combination with 100 ng / ml anti-CD3 antibody. These concentrations of antibody were chosen for the expansion and maintenance of T cell lines without the use of feeder cells.

2.4.6 Phenotype of cells grown on antibody coated plates.

Long-term culture of T cells on anti-CD3 antibody coated plates promotes the expansion of $CD8^+$ T cells. Since the objective was to isolate $CD4^+$clones, the preferential expansion of $CD8^+$ T cells was undesirable. Therefore it was necessary to determine whether there was selective expansion of $CD8^+$ cells when T cells were

cultured on anti-CD28 and anti-CD3 antibody coated plates. Figure 2.9 shows the long term effect on phenotype of T cells maintained on antibody coated plates.

Figure 2.9 Phenotype of T cells after long-term culture on antibody coated plates



■ = $CD4^+$, □ = $CD8^+$, ▲ = $CD25^+$. The phenotype of PBMC from 2 patients cultured for 40 days (as described in section 2.3.6) on anti-CD3 and anti-CD28 antibody coated plates is shown. Samples of cells were removed at various time points and analysed for the expression of CD4, CD8 and CD25 on the cell surface by flow cytometry described in section 2.3.6.

The results shown in Figure 2.9 show that PBMC from patient A initially had 30 % $CD4^+$, 23 % $CD8^+$ and 1% $CD25^+$ cells. Similarly patient B had 40% $CD4^+$, 25 % $CD8^+$ and 5 % $CD25^+$ cells. A rapid expansion in the percentage of the $CD4^+$ population occurred within the first few days of culture. Patient A's $CD4^+$ T cells were 52% of the total population by day 15, and patients B's $CD4^+$ cells were 68 %.

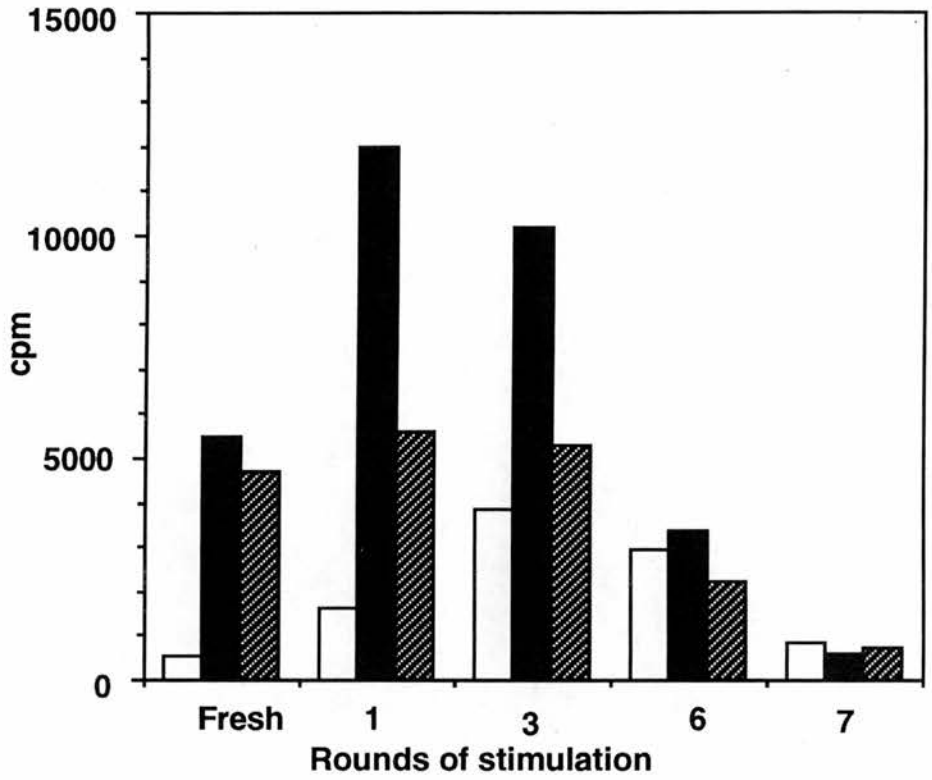
Similarly, $CD25^+$ cells increased to 81% (patient A) and 84% (patent B) during the first 15 days of culture. It is likely that many of the $CD4^+$ T cells were also $CD25^+$. The numbers of $CD4^+$ T cells were maximal between day 10 and 15 of culture, although after this time the percentage decreased markedly. By day 20 the percentage of $CD4^+$ T cells within the culture had fallen to less than 10% of the total population, (shown in both subjects). The $CD8^+$ T cells showed a less rapid expansion, peaking to 70% of the total cell population (patient A), and 71% (patient B) by day 34 of culture. The viability of the cells remained > 80% for both patients. However, at the last two time points the viability had fallen to 66% and 88% for patient A, and 55% and 60% for patient B.

2.4.7 HPV-specific T cell responses from an oligo clonal cell line

Freshly isolated PBMC from 46 individuals were screened for their lymphoproliferative responses to HPV, as shown earlier in Figure 2.6. Many of these PBMC were also expanded on anti-CD28 : anti-CD3 antibody coated plates before screening for responses to HPV. As APC were not present in the T cell cultures either autologous or allogeneic APC were added to these assays. A total of 14 cell lines from different individuals were screened for proliferative responses to HPV. Only one cell line showed a response to antigen and this was from a patient with current HPV-2 infection. Lymphoproliferative responses of freshly isolated PBMC from donor 15 are shown in Figure 2.10 in comparison with the response from a cell line at different passage number established from donor 15.

Figure 2.10 shows an increased cpm in response to HPV after one round of stimulation on coated plates compared with freshly isolated PBMC. Although due to a higher background cpm after culture, the SI was reduced from 9.8 in freshly isolated PBMC to 7.3 after 1 round of stimulation. After seven rounds of stimulation the response to HPV was reduced from a high response (SI = 7.3) to no response (SI = 0.7).

Figure 2.10 Responses to HPV from PBMC or oligo clonal cell lines



□ = medium only, ■ = HPV-1 , ▨ = HPV-2. The PBMC from patient 15 were cultured on anti-CD3 and anti-CD28 antibody coated tissue culture plates. The response of the oligo clonal cell line to HPV-1 and 2 was screened after several rounds of stimulation and compared to freshly isolated PBMC. One round of stimulation was equivalent to 7 days stimulation on a fresh antibody coated plate in the presence of 20 IU / ml IL-2. SEM < 29% mean of triplicates.

2.4.8 Cloning experiments.

After screening a range of donors, several of these were chosen to proceed with cloning. Table 2.1 shows results from seven cloning experiments using 6 separate donors. PBMC from 2 different donors (donors 19 and 37) gave positively proliferating cultures when visualised by inverted light microscopy. However, all of these positive cultures failed to further expand *in vitro* in the presence of APC, antigen and IL-2.

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Table 2.1 Results of cloning experiments

Donor	26 fresh	2 cell line	AS fresh	19 fresh	19 fresh	37 cell line	38 cell line
length of primary stimulation	9 days	6 x rounds	9 days	2 x 9 days	2 x 9 days	7 days	7 days
separated blasts with	percoll	percoll	ND	Lympho prep	Lympho prep	ND	Lympho prep
cloning wells	20 µl wells	20 µl wells	20 µl wells	20 µl wells	20 µl wells	200 µl wells	200 µl wells
positives at 30 cells / well	0 / 60	0 / 300	0 / 300	7 / 100	10 / 100	10 cells / well = 4 / 28	10 cells / well = 0 / 20
3 cells / well	0 / 60	0 / 300	0 / 300	4 / 100	4 / 100		
0.3 cells / well	0 / 60	0 / 300	0 / 300	1 / 100	1 / 100		
successful expansion	none	none	none	none	none	none	none

Table 2.1 Fresh = PBMC which were freshly isolated prior to stimulation with HPV for 9 days and then cloned from this population. Cell lines = PBMC which were stimulated with HPV for 9 days and then expanded on anti-CD3 anti-CD28 antibody coated tissue culture plates before cloning. The duration of culture before cloning is indicated. T cell blasts which had been isolated by percol, lymphoprep™ or not separated but counted (ND), were seeded at 30, 3 or 0.3 cells per well in Terazaki plates with APC, antigen and IL-2. In some cloning experiments (donors 37 and 38) 10 blasts per well were seeded in 200 µl wells. During the following 14 day period the cultures were scored visually as positive or negative relative to proliferation for each cell density. The frequency of responding cultures is shown in each box.

2.5 Discussion

Infection with HPV is chronic in nature and can persist even in an immunocompetent host, suggesting an effective evasion strategy of the virus or a defect in the elicitation of an effective immune response. Thirty percent of warts regress within 6 months (Bunney *et al* 1992) and it is likely that these patients generated effective T cell responses towards the virus at that time. In order to design effective vaccines and therapies it is critical that the nature of this CMI response towards HPV is understood. Investigations into CMI towards HPV have mainly concentrated on infections with the mucocutaneous types of HPV, the oncogenic types of which can lead to carcinoma (frequently HPV-16 and 18). Many of these studies have been undertaken in mice and fewer studies have investigated CMI to cutaneous HPV types in humans. The following discussion will cover possible reasons for poor lymphoproliferative responses obtained to purified HPV *in vitro,* and will then discuss findings from other studies on responses to other HPV ORFs in humans and mice.

2.5.1 Clinical history of HPV infection

Previous studies on T cell responses from HPV infected individuals have suggested that the degree of *in vitro* T cell proliferation to HPV is related to the clinical status of the patient (*i.e.* individuals with resolving warts or those who are asymptomatic, have higher responses than people with warts of several years duration) (Steele *et al* 1993). However, not all studies reached the same conclusion (Charleson *et al* 1992). This hypothesis is difficult to investigate, because the true clinical status of an individual with regard to cutaneous HPV infection cannot be accurately determined, due to the lack of a pre-clinical diagnostic test. The majority of people have had cutaneous warts, indeed 85% of normal laboratory donors have antibodies to HPV-1 (Steele *et al* 1990). If a T_H cell response is generated *in vivo* during regression of warts these individuals will have circulating HPV-specific memory T cells.

Individuals without current HPV infection (for use as controls) may be asymptomatic but are unlikely to be naive with respect to HPV infection. These individuals may have been infected with HPV which could remain latent before the infection shows clinical signs and the consequent change in epidermal morphology. Alternatively, HPV DNA may remain latent after the resolution of a wart. Patients with HPV infection in this study had their warts for at least 1 year and most were non-regressing at the time they volunteered the blood. PBMC were also isolated from patients whose warts had completely resolved or were in the process of resolving.

2.5.2 Purified HPV as an antigen

Antigen is required in large amounts to clone and maintain antigen specific T cells. Due to the lack of a viable culture system to obtain sufficient purified virus, HPV was obtained from clinical material for this study. The fact that HPV cannot be propagated in conventional virus titre assays *in vitro* prevents the accurate quantification of the number of particles present in each preparation. Four methods were used to standardise batches of HPV, and to characterise the antigen. Transmission electron microscopy was essential to confirm the presence of virus particles and showed qualitative differences in amount of virus in different batches. The Biorad protein estimation was quantitative but a high virus titre was required to be within the range of the assay. SDS-PAGE was used to characterise the purified HPV and silver staining of 3 different batches indicated many protein species present in all three preparations. The main bands visible were 78 kD, 66 kD and 55 kD in size. The 78 and 66 kD bands may be the L2 structural protein, while Western blot confirmed that the 55 kD was the L1 (Doorbar *et al* 1987), although the presence of contaminating host cell keratins cannot be ruled out. The three batches were prepared from samples clinically diagnosed as either HPV-1, HPV-2 or undiagnosed cutaneous viral warts. However, HPV-1 L1 was detected in all three batches to different extents. This indicates that determining the HPV type based on

batches to different extents. This indicates that determining the HPV type based on clinical observations is inaccurate In order to standardise the different batches of purified HPV, the virus was used as antigen at a range of concentrations in lymphoproliferation assays. The concentration of virus generating optimal proliferation of PBMC was determined by Biorad protein estimation to be 2 μg / ml.

2.5.3 Status of HPV infection and proliferative response

In order to generate T cell clones to purified HPV, many individuals with or without current HPV infection were screened for lymphoproliferative responses to purified antigen. Great variation existed in lymphoproliferative responses to purified HPV within a given group of individuals. In general, lymphoproliferation in response to purified HPV was low (SI< 5) although PHA responses were high (data not shown). Similarly Cubie *et al* (1988) measured low lymphoproliferative responses to purified virus from patients attending colposcopy clinic, where the majority of SI were < 2. Although 29 % of patients with current warts responded with an SI >3, only 8% individuals with no current warts responded to the virus. However, no significant difference was found between the percentage of responders with or without current cutaneous warts. These findings do not support the hypothesis that asymptomatic individuals have greater proliferative responses than those with current HPV infection. This in part may be due to the low number of individuals without current HPV infection tested in this study. Asymptomatic individuals as a group may also include people with warts which have recently regressed, with warts unknown to them or with a subclinical infection. Thus the division between these two groups is not very distinct. PBMC from individuals with plane warts did show a significantly greater ($p = 0.02$) proliferation than individuals without current HPV infection. It is possible that infection with HPV types associated with plane warts (HPV-3 and 10) induce T cell responses to a greater extent than infection with other cutaneous types. However, since only 4 subjects with plane warts were analysed, more subjects need to be screened before conclusions regarding this can be made. PBMC from donors

whose warts were regressing at the time blood was taken showed no difference in proliferation when compared with PBMC from individuals whose warts showed no change in status. However, one individual whose wart had regressed within 1 month of the analysis responded with an SI of 36. This finding supports the evidence that *in vivo* proliferative responses towards HPV may only be transient. Kadish *et al* (1994) followed the PBMC response of one patient whose warts underwent spontaneous regression. During regression a proliferative response was observed, but when assayed 3 months later, the same response could not be demonstrated.

The lymphoproliferative responses to purified HPV reported in this study are greater than described previously (Charleson *et al* 1992). Charleson *et al* (1992) observed 32 % patients and no controls with a proliferative response of SI > 2 to purified HPV-1 and 2. In the present study a greater amplitude of response was observed in many patients with the highest SI of 36, and many SI between 2-5. If the same criteria were used as Charleson *et al* (1992), 41% of patients either with or without current HPV infection would have positive proliferative responses in this study. The present study showed a similar range of SI (0 -11) as seen previously, although Steele *et al* (1993) found a greater percentage of asymptomatic individuals were found to respond with an SI > 3 (52%). Cubie *et al* (1988) observed that 25% colposcopy patients responded to purified HPV-1 and 2 (which are types not associated with genital HPV infection). In addition there was no correlation with the degree of dysplasia.

2.5.4 HPV type and proliferative response

In this study, no correlation could be made between type of wart and proliferative response. Since HPV DNA typing was not available, patients were divided into groups having either common, plantar or plane warts on clinical and histopathological observations. Although plantar warts are often caused by HPV-1, common warts by HPV-2 and plane warts by HPV-3, other types of HPV are found

in these cutaneous lesions. DNA typing is essential to reach a definitive conclusion regarding correlation between HPV type and proliferative response.

Since immune responses to HPV may be type specific the possibility of using the incorrect HPV type as antigen cannot be ruled out, and may explain the lack of response in some individuals. Indeed one study observed that regression of HPV-3 plane warts does not cause simultaneous regression of common warts in the same patient (Tagami *et al* 1980). Cubie *et al* (1988) found positive responses of PBMC from patients attending a colposcopy clinic to purified HPV-1 and HPV-2 virus (usually cutaneous types). This response correlated with the patient history of skin warts and not the grade of dysplasia due to HPV-16 infection. However, Strang *et al* (1990) generated T cell clones reactive against five determinants from HPV-16 L1. T cell clones specific for three of these determinants were cross reactive with peptides corresponding to the L1 peptides from other HPV types. Kadish *et al* (1994) identified T cell responses to HPV-16 E7 peptides which appear to be HPV-16 group specific, since 64% of patients with HPV-16 (or the closely related HPVs 31 and 33) responded to the peptides whereas only 16% of patients with other HPV infections responded. This suggests that a type specific (or closely related type) response may occur *in vivo*. Taken together the present evidence indicates that some cross reactivity exists in immunity to HPV types and is more likely to occur between similar HPV types.

2.5.5 HPV could be immunosuppressive in vitro

Whole HPV could exert a suppressive effect within the culture: HPV itself may interfere with T cell proliferation *in vitro*. This is the case with Herpes Simplex virus which may prevent monocytes from providing necessary co-stimulation for T cell proliferation (Hayward *et al* 1993). Alternatively it is possible that HPV may induce soluble suppressive factors *in vitro* (Chopra *et al* 1991) and suppression caused by HPV could reflect an evasion strategy *in vivo*.

2.5.6 Antigens not present in purified HPV

Proteins present in the whole inactivated HPV may not be the appropriate antigens to elicit an immune response. Peptides provided by purified virus e.g. structural proteins of the virus such as L1 and L2, may not be antigenic *in vivo*, therefore little response would be seen upon secondary stimulation *in vitro*. Other proteins encoded by the virus which do not form a structural part of the virion itself (e.g. E4, E6, E7) may be the antigens inducing the immune response *in vivo*.

2.5.7 Epithelial sequestration of antigen

HPV is exclusively an intraepithelial pathogen which replicates in KC of the differentiated upper layers of the epidermis and is released when they slough off the top of the epidermis. LC present in the epidermis migrate to the draining lymph node after stimulation by antigen or insult such as UV light or contact sensitisers (Moodycliffe *et al* 1992; Macatonia *et al* 1986; Moll *et al* 1993). In HPV infection, antigens may not be released by KC into the epidermis, thus viral antigen may be sequestered away from the main APC of the skin. Therefore, if T cells are not presented with HPV antigen *in vivo*, a secondary response will not be observed *in vitro*, resulting in the poor responses seen in this study.

2.5.8 Local response versus systemic response

If a local HPV-specific immune response is generated, then venous blood may be an inappropriate source to screen for responses. Primed T cells may occur at such low frequency in peripheral blood that an *in vitro* response cannot be measured. HPV-specific T cells may home back to the site of infection where greater numbers accumulate.

2.5.9 Reduction of antigen presentation by Langerhans cells

LC are found in reduced density in the majority of cutaneous warts (Chapter 3 and Hughes *et al* 1988; Viac *et al* 1992a; Chardonnet *et al* 1986; Chardonnet *et al* 1983).

It is not clear whether LC initially migrate from the epidermis during HPV infection and are not replaced with LC from progenitors in the blood, or if HPV infection occurs as a result of reduced LC density in the epidermis. If the latter occurred, the reduced density of epidermal LC at the site of infection could lead to T cells not being exposed to antigen. However, it has been observed that some chronic warts have no reduction in epidermal LC (Chapter 3), which may suggest that a reduced LC density alone cannot explain persistence of HPV infection. It is possible that warts which do not have a reduced density of LC were about to regress, since an infiltration of dendritic cells is observed in regressing plane and genital warts (Fierlbeck *et al* 1989).

2.5.10 Lack of MHC class II expression on keratinocytes

Resident KC in the epidermis do not express MHC class II (Viac *et al* 1993) but can be induced to express these molecules (Coleman *et al* 1994a). The up-regulation of MHC class II on KC by γ-IFN implies that KC can serve as APC for T cells. Presentation of herpes Simplex virus by MHC class II $^{+}$ KC to T cells *in vitro* has been demonstrated (Cunningham *et al* 1989). However, MHC class II expression on KC is not seen in the epidermis of cutaneous warts (Viac *et al* 1993) although it has been detected at low levels in benign laryngeal papillomas and low grade genital lesions (Viac *et al* 1993; Viac *et al* 1992b; Norton *et al* 1991). Lack of MHC class II on KC may be partly responsible for poor lymphoproliferative responses observed *in vitro* to HPV antigens. Regression is associated with the expression of MHC class II on epidermal KC in genital (Coleman *et al* 1994a; Fierlbeck *et al* 1989) and plane (Aiba *et al* 1986) warts. MHC class II induction on KC may be necessary for an HPV-specific immune response to occur. MHC class II is frequently found in high grade cervical intraepithelial neoplasia (Coleman *et al* 1994b; Glew *et al* 1992). However, Glew *et al* (1992) found no correlation between MHC class II expression and the presence of HPV-16 DNA in cervical squamous cell carcinomas.

2.5.11 MHC Class I modulation

MHC class I molecules are expressed constitutively on most somatic cell types and are responsible for the presentation of peptide fragments of viral proteins to $CD8^+$ T cells. A reduction in the expression of MHC class I is frequently observed in cervical carcinoma (Connor *et al* 1990), but this decrease does not occur in all HPV induced carcinomas, indicating no direct effect of HPV on MHC class I expression. MHC class I reductions are most prominent in invasive cervical carcinomas, whereas no evidence of class I modulation is observed in benign HPV associated disease, low grade cervical intraepithelial neoplasia or cutaneous warts (Viac *et al* 1990). Modulation of class I MHC may enable HPV induced carcinomas to evade the immune system although it is not a strategy utilised in benign HPV infections.

2.5.12 Clonal anergy

Induction of MHC Class II expression on KC allows these cells to function as APC. Presentation of Herpes Simplex virus (Cunningham *et al* 1989), bacterial derived superantigens, PHA, anti-CD3 (Nickoloff *et al* 1993), and *M. leprae* antigens (Mutis *et al* 1993) by class II^+ KC to $CD4^+$ T cells has been observed *in vitro*. However, the presentation of alloantigen (Nickoloff *et al* 1993), influenza virus peptides (Bal *et al* 1990), and contact allergens (Gaspari *et al* 1988) by MHC class II^+ KC *in vitro* and *in vivo* (Gaspari *et al* 1991) have been shown to induce a non-responsive or anergic state. Anergy occurs when peptide and MHC II are presented to T cells without co-stimulation by APC. Important receptors involved in costimulation are the B7 group (B7.1, B7.2, B7.3) which interact with CD28 or CTLA-4 on T cells (Nickoloff *et al* 1994). KC express B7.3 which binds to CD28 but does not provide co-stimulatory signals (Nickoloff *et al* 1994). Non-regressing warts have been shown to lack MHC class II on KC (Viac *et al* 1993), therefore KC are not able to present antigen to $CD4^+$ T cells. However, if expression of MHC class II on KC is required for regression to occur, then the lack of co-stimulatory molecules such as B7.1 or B7.2 to stimulate CD28 on the surface of the T cell could induce anergy.

MHC class II is induced in regressing genital HPV lesions (Coleman *et al* 1994a), but it is unknown whether this is a necessary requirement for wart regression. It is also unknown if co-stimulatory molecules are also upregulated on the KC of regressing warts. Transfection of B7 into tumourigenic cell lines which already possess HPV-16 E7 leads to regression of the tumours when these cell lines are inoculated into immunocompetent mice (Chen *et al* 1992).

Other molecules which may be involved in cutaneous co-stimulation are ICAM-1, LFA-3, heat stable antigen (Chen *et al* 1993) and the invariant chain (Ii-CS) which associates with MHC class II and is thought to enhance T cell responses via the CD44 molecule (Naujokas *et al* 1993).

Low levels of HPV antigen presented to the immune system may also be a cause of anergy. This has been demonstrated in a mouse model where high levels of antigen presented to the immune system induce an immune response while low levels induce an anergic state (Chambers *et al* 1994).

2.5.13 Decreased general cell mediated immunity

Although lymphoproliferation was generally poor in response to whole virus, the PBMC did proliferate to mitogens such as PHA (data not shown). This indicates that PBMC from individuals tested were still able to mount T cell responses which is in agreement with some studies (Vardy *et al* 1990; Androphy *et al* 1984) but not others (Carson *et al* 1986; Obalek *et al* 1980).

2.5.14 Cloning experiments

In this study, individuals who showed initial responses to purified HPV were used for cloning experiments. Cloning was attempted either from freshly isolated PBMC which had undergone stimulation with purified HPV for 9 days, or PBMC which had undergone initial stimulation with purified HPV and then subsequent growth on

tissue culture plates coated with antibodies. The advantage of culturing T cells *in vitro* before cloning are that T cells are selectively enriched on anti-CD3 coated plates, and after the first round of stimulation, are not under any negative influence which may be present in a culture of whole PBMC (e.g. APC, HPV or other T cells). Culture of lymphocytes without APC may increase the success of subsequent cloning by either removing negative influences or by increasing the frequency of antigen specific precursors. Indeed, one investigation isolated melanoma-specific CTL clones from cultured PBMC but could not elicit CTL from fresh PBMC from the same patient (Mukherji *et al* 1989).

2.5.15 Growth of T cells on antibody coated plates.

Anti-CD3 and anti-CD4 antibody coated plates were used to investigate the potential of CD3 and CD4 as co-stimulatory ligands for expansion and maintenance of T cells prior to cloning. Co-stimulation via CD4 molecule did not augment T cell proliferation by anti-CD3 alone and was not used further. Anti-CD28 antibodies were used in conjunction with anti-CD3 antibodies as they generated increased proliferation compared with anti-CD3 alone. These antibody coated plates maintained $CD4^+$ cells over short periods of time (15 days). Lymphoproliferation towards HPV was increased after one round of stimulation on coated plates, but after seven rounds of stimulation the response had diminished markedly. These results suggest that the coated plates are of use in the maintenance of these cell lines but only in the short term (< 2 rounds). Long term culture on these plates (30 - 40 days) preferentially selected $CD8^+$ cells. Fourteen cell lines derived from donors with different clinical history were expanded on anti-CD3 and anti-CD28 antibody coated plates before being screened for responses to whole HPV using autologous APC. A mixed pool of 3 allogeneic donors were also used to increase the chance of presentation by an APC of matched HLA type. Only a very small proportion of these donors responded to purified HPV (1/14), indicating that the strategy of expanding T cells on coated plates was not augmenting the HPV specific response.

Cloning by limiting dilution was attempted from either these expanded cell lines or from fresh PBMC. The PBMC from one donor produced positive cultures, but these positive cultures failed to expand when they were transferred to larger wells with APC, antigen and IL-2. The failure to obtain T cell clones could be due to several reasons:- 1) there were no antigen specific T cells responding and colonies picked as positives by eye were not genuine proliferating cultures, 2) the method was inappropriate for selecting low frequency precursor T cells, 3) expansion of clones was not optimal. 4) antigen was inappropriate and 5) APC were inappropriate.

There may be ways of improving the success rate of cloning. After initial culture with antigen other cytokines such as IL-12, or IL-4 could be added to the culture to stimulate either T_H1 or T_H2 T cell clones respectively. Alternatively antibodies towards these cytokines may promote clonal growth of T cell subsets. PBMC from a panel of HLA typed donors to use as APC would remove the limitations of obtaining sufficient APC from one particular donor. Other HPV antigens may be more appropriate to generate recall responses *in vitro*.

2.5.16 Human T cell responses to HPV E4

In order to identify epitopes involved in immune responses to HPV, researchers have generated overlapping peptides derived from HPVs and identified sequences which produce T cell responses *in vitro*. In addition, peptides which can potentially induce immune responses have been determined by screening their binding to MHC molecules. Alternatively, predictions have been based on elution of peptides from MHC antigens or by using motifs of known T cell epitopes (Rothbard *et al* 1988; De Lisi *et al* 1985).

Steele *et al* (1993) screened individuals for HPV specific lymphoproliferative responses and generated T cell clones from one donor whose PBMC responded to both HPV-1 and native HPV-1 E4. However, in these studies it was not clear if

cloning was attempted for whole virus. Using HPV-1 E4-specific T cell clones, Steele *et al* (1993) determined which part of E4 was recognised by screening responses to 15-mer overlapping peptides derived from the E1^E4 protein. The minimal epitope recognised by one clone was a 13 mer peptide (aa 38 - 50) and did not fit predictions from De Lisi *et al* (1985) or Rothbard *et al* (1988). Blocking studies suggested that this peptide was restricted by an identified HLA-DQ antigen.

Although responses to native E4 protein were detected (Steele *et al* 1993), responses to HPV-1 E4 β-gal fusion protein were obscured by proliferation towards β-gal alone (Charleson *et al* 1992). Cubie *et al* (1989) also found little evidence of HPV-specific T cell responses to β-gal-HPV-16 E4 fusion proteins in a 6-7 day proliferation assay. However, β-gal fusions with other antigens have been successful in generating antigen specific T cell responses (Shepherd *et al* 1994).

The lymphoproliferative response towards HPV E4 observed by Steele *et al* (1993) suggests previous immune recognition of E4. However, without analysis of memory and naive populations this remains speculative since *Plasmodium falciparum* specific T cell responses have been demonstrated from non-exposed individuals. The responses from non-exposed individuals may be due to either cross reactive memory T cells (Currier *et al* 1992), or in some cases to naive T cells (Fern *et al* 1992). The T_H response to HPV-E4 may be important in immunity against HPV-1 infections such as deep plantar warts where this antigen is highly abundant.

2.5.17 Humans T cell responses to HPV E7

E7 is the most abundant viral protein in HPV-16 associated genital cancer and has been shown to have transforming activity *in vitro*. As a result, many studies have investigated HPV-16 E7 specific T cell responses and attempted to map the epitopes involved in recognition. One approach has been to generate overlapping 17-26 mer (Kadish *et al* 1994) and 14 mer (Altman *et al* 1992) peptides which span the E7

region and use these to identify epitopes involved in generating T cell responses. Altman *et al* (1992) generated $CD4^+$ T cell lines from asymptomatic seropositive individuals, which responded to HPV-16 E7 peptides. Three determinants of E7 were recognised in association with two MHC class II types and some of these $CD4^+$ clones had cytolytic activity. One E7 peptide (amino acids (aa) 17-39) containing a T_H epitope identified by Altman *et al* (1992), was also identified by Kadish *et al* (1994). Kadish *et al* (1994) did not observe proliferative responses to HPV-16 E7 peptides after 7 days, but by 21 days in bulk culture with the addition of IL-2 responses were evident. Twenty eight percent of patients attending a colposcopy clinic and 23% of controls responded to at least one of these peptides. It may be the case that the precursor frequency for these antigens is low *in vivo* and extended culture causes expansion of precursors. Alternatively, T cells may have been primed *in vitro* to E7 peptides during the extended initial culture. If T cells were primed *in vitro*, then the proliferative responses would not reflect *in vivo* responses. This seems unlikely as the T cell responses to one peptide were related to current cervical infection with HPV-16 and the closely related HPV-31 and HPV-33 types.

2.5.18 Human T cells responses to other HPV proteins

Shepherd *et al* (1994) identified immunodominant regions of the HPV-16 L1 ORF using T cell lines derived from colposcopy patients. Fusion proteins of β–gal HPV-16 L1 were used to generate L1-specific T cell lines by culture for 14 days. These were then screened for responses to L1 peptides. Although poor responses were found to peptides, 3 immunodominant regions of the L1 were identified. Strang *et al* (1990) demonstrated four T_H determinants in L1 and generated human T_H cell clones specific for HPV-16 L1 peptides predicted from algorithms of MHC class II binding T cell epitopes (Rothbard *et al* 1988). One T_H determinant was found in HPV-16 E6 which generated *in vitro* proliferative responses (Strang *et al* 1990), and Cubie *et al* (1989) found proliferative responses to HPV-16 or 18 E6-β-gal fusion proteins from

29% patients with cervical intraepithelial neoplasia, but the majority of SI were < 3.

Although HPV-specific CTL have been demonstrated in mice (Stauss *et al* 1992; Sadovnikova *et al* 1994), Tarpey *et al* (1994) were the first to demonstrate human HLA-A2 restricted CTL specific for HPV-11 E7 peptides. However, this may not reflect the presence of HPV specific CTL *in vivo* since it is possible that the CTLs were primed *in vitro*.

2.5.19 MHC restriction

The T cell response to a protein is limited to a few epitopes which in turn is restricted by the HLA type of the host. Individuals that express HLA types which do not bind immunogenic epitopes of HPV are presumably more susceptible to long term infection with HPV. Indeed, an association of HLA-DQw3 and cervical carcinoma has been identified in HPV-16 infections (Wank *et al* 1991). The MHC class II of the host has also been shown to be important in the regression of warts caused by cottontail rabbit papillomavirus (CRPV), where the rejection of warts was related to DR and DQ genotypes (Han *et al* 1992).

In humans HLA-B7 genotype is also associated with poorer clinical outcome for cervical carcinoma patients (Keating *et al* 1995). This is partly mediated by loss of HLA-B7 expression which correlates with metastasis or cervical neoplasia. In HLA-B7 individuals a variation in HPV-16 E6 was demonstrated (Ellis *et al* 1995). This E6 variant was observed in an epitope which was shown to bind MHC class I *in vitro*. Thus an escape mechanism from immunosurveillance by HPV-16 E6 specific CTL occurs in individuals with HLA-B7 haplotype. In this respect the modulation of class I expression also has an effect such that 73% of cervical carcinomas had a reduction in expression at HLA-A or B alleles. Thirty eight percent of these also had a decrease in the transporter associated with antigen presentation -1 (TAP-1) (Keating *et al* 1996), indicating that TAP-1 expression may

be partly responsible for the observed reduction in HLA-A or B expression. Polymorphisms in the TAP genes also influence the spectrum of peptides presented by MHC class I (Powis *et al* 1992), which may contribute to genetic susceptibility or protection.

2.5.20 Murine T_H epitopes

Although papillomavirus is species specific and HPV does not infect mice under natural conditions, the antigenicity of HPV proteins has been determined in murine models. Tindle *et al* (1991) demonstrated a murine T_H epitope of HPV-16 E7 (contained within aa 48-57) in 5 mice strains with differing class II MHC and was not predicted using the predictions of Rothbard *et al* (1988) or De Lisi *et al* (1985). This region has been confirmed as containing a T_H epitope both in humans (Kadish *et al* 1994) and mice of differing H-2 haplotypes (Comerford *et al* 1991; Shepherd *et al* 1992).

Three other murine T cell epitopes were found in HPV-16 E7. Amino acids 20-29, 69-80 and 85-90 were identified as containing T_H epitopes (Comerford *et al* 1991; Shepherd *et al* 1992; Ressing *et al* 1995) and CTL epitopes in mice (Sadovnikova *et al* 1994; Bauer *et al* 1995). In addition, amino acids 69-80 and 85-90 were confirmed as TH epitopes in humans (Kadish *et al* 1994; Altman *et al* 1992).

HPV-16 E7 specific responses were also demonstrated in a mouse model in which E7 was presented to the immune system at the epidermis by grafting an HPVE7 expressing cell line onto syngeneic mice. *In vitro* lymphoproliferation towards E7 was found in recipient mice challenged with a vaccinia recombinant expressing E7 (Chambers *et al* 1994). These experiments emphasise the importance of E7 in infections with HPV-16 where it is expressed in abundance. Although two H-2^k restricted T_H epitopes were found in HPV-18 E7 (Fernando *et al* 1995), a public epitope of E7 similar to the HPV-16 E7 (aa 49-57) was not found in HPV-18 E7.

2.5.21 Murine CTL epitopes

The HPV-16 E7 peptide aa 49-57 shown to contain a T_H determinant also contained a murine CTL epitope restricted by H-2D^b and K^b (Sadovnikova *et al* 1994; Bauer *et al* 1995). In addition D^b mice immunised with a peptide containing this determinant were protected against challenge with HPV-16 transformed tumourigenic lines (Feltkamp *et al* 1994). Stauss *et al* (1992) demonstrated murine CTL responses to 15/20 peptides derived from HPV-16 E7, E6 or L1 which bound to class I MHC. Not all of these sequences were predicted by known motifs of naturally processed peptides. Sadovnikova *et al* (1994) also used murine MHC class I binding to predict E7 peptides containing CTL epitopes, and compared these predicted epitopes to naturally processed peptides eluted from E7 expressing cells. Not all peptides found to be naturally processed were predicted epitopes, and some CTL generated from predicted epitopes did not recognise E7 expressing cells. This suggests that natural peptide epitope mapping is more appropriate than synthetic peptide epitope mapping using predictions and HLA binding assays. This approach is not possible in all studies since eluting peptides from infected cells requires large numbers of cells and the sequence of the antigen must be known. However, Falk *et al* (1991) provide evidence that epitopes from natural peptides are identical to those from synthetic peptides, indicating that both methods are appropriate for epitope mapping.

2.5.22 Conclusion

The data presented in this chapter demonstrate that some donors have lymphoproliferative responses to purified HPV *in vitro*., but these responses were not related to status of the infection of the donor. The proliferative response of these individuals may reflect immunity to HPV *in vivo* at that time. However, HPV-specific T cell clones could not be generated from these donors.

Chapter 3

Local immune responses in cutaneous warts

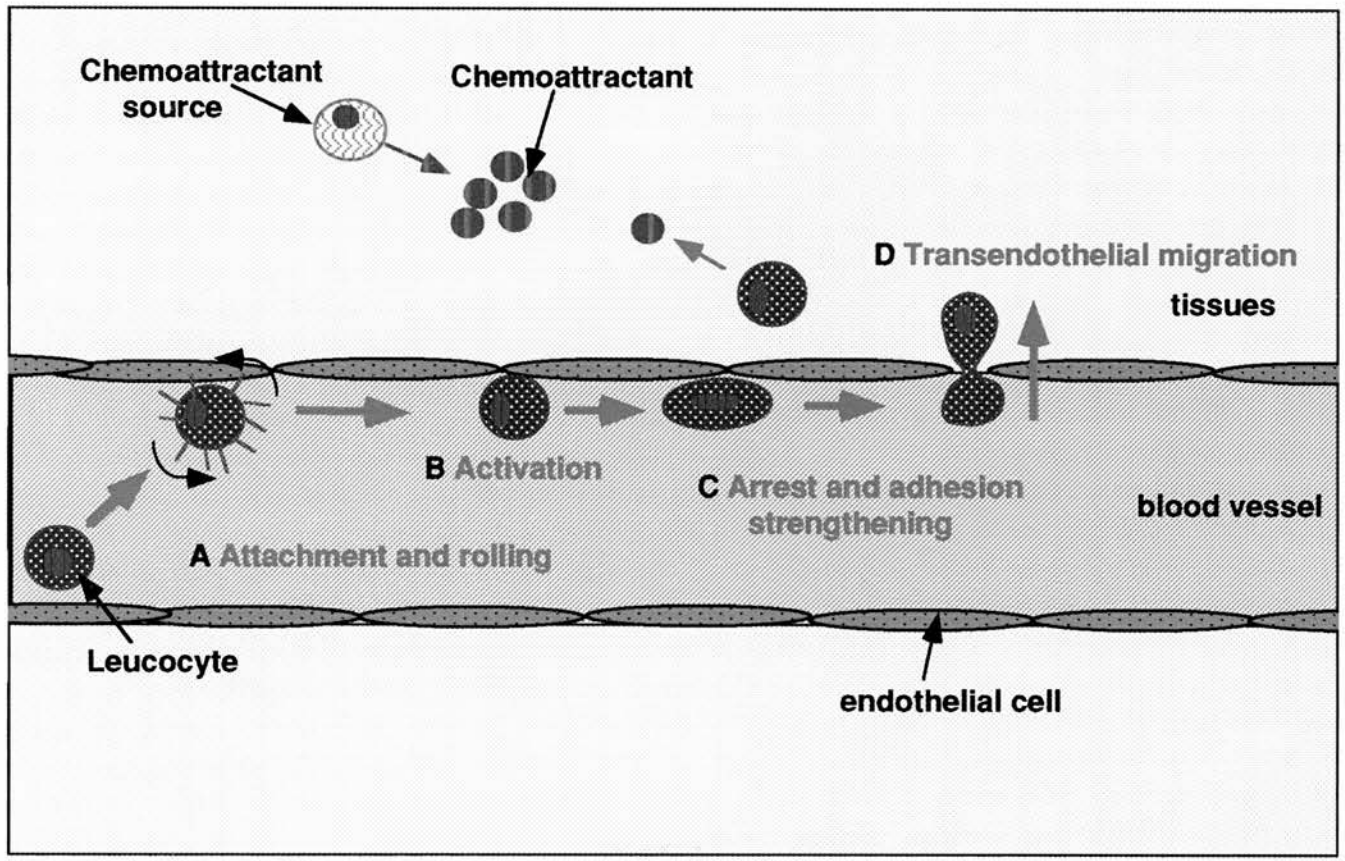
3.1 Introduction

The previous chapter examined systemic T cell responses towards purified HPV in lymphoproliferation assays. HPV specific T cell clones could not be generated and 80 % of patients who had current HPV infection with no signs of regression did not show proliferation towards HPV. One explanation for poor T cell responses to HPV discussed in chapter 2 is that modulation of immune functions by HPV may occur within the wart rather than in peripheral blood, therefore, this chapter demonstrates investigation of local immunity in cutaneous warts.

3.1.1 Lymphocyte trafficking

Leucocyte adhesion and trafficking have been excellently reviewed by Springer (1994) and Hogg (1993). Lymphocytes constantly recirculate from the blood into the tissues and back into the lymph and blood, in a process which is part of the normal surveillance for foreign pathogens. The migration of leucocytes into tissues can be dramatically increased in an inflammatory reaction. Immune effector cells enter tissues at postcapillary vascular sites where the specialised endothelial cell walls have similarities to high endothelial venules associated with migration into lymphoid organs. Adhesion molecules on endothelial cells mediate trans-endothelial migration and their differential expression determine which subsets of leucocytes cross the endothelium. Cytokines within the local environment influence the expression of vascular adhesion molecules, thus the nature of the inflammatory stimulus will dictate the type of cells entering the tissues. The movement of leucocytes through the vessel walls into the tissues occurs in four overlapping steps shown in Figure 3.1.

Figure 3.1 The multi step model of leucocyte migration



Taken from Springer T.A. (1994) Traffic signals for lymphocyte recirculation and leucocyte migration. Cell 76 p 301-314

First, leucocytes loosely attach to the vessel wall via selectins and begin rolling in the direction of the blood flow (Figure 3.1 A). Selectins are a family of carbohydrate binding molecules; L-selectin is expressed on the majority of circulating leucocytes which bind to ligands on inflamed endothelium, while P-selectin and E-selectin are induced on activated endothelial cells which bind to ligands on passing leucocytes. The sialyated form of the Lewis X antigen has been shown to be an important determinant in the binding of the selectins (Vestweber, 1993). Rolling of leucocytes along the endothelium has been attributed to rapid rates of binding and dissociation of selectins (Lawrence *et al* 1991) although the rapid shedding of L-selectin after binding may contribute to this effect. In addition to the selectins, the $\alpha 4$ integrins expressed on leucocytes can initiate rolling on mucosal addressin (MadCAM-1) and VCAM-1 expressed on endothelium (Berlin *et al* 1995; Jones *et al* 1994).

In the second phase, activation of integrins on these loosely attached rolling leucocytes (Figure 3 B). Integrins are hetero-dimers of an α subunit non-covalently linked to a β subunit. At least 14 different α, and 8 different β subunits have been identified, thus forming at least 20 different integrin hetero-dimers which are differentially expressed on leucocytes. T cells express LFA-1 (α_1 β_2 or CD11a, CD18) and very late activation antigen-4 (VLA-4) ($\alpha_4\beta_1$ or CD49d, CD29). Neutrophils express LFA-1 and MAC-1 ($\alpha M\beta 2$ or CD11b, CD18), monocytes express LFA-1 and all leucocytes express p150,95 ($\alpha X\beta 2$ or CD11c, CD18). Many integrins including LFA-1 require activation before binding to their ligand can occur (Dustin *et al* 1989). Chemokines may provide the activation signal for integrins since IL-8 and macrophage inflammatory protein-1 β (MIP-1β) can transiently trigger the activation of integrins such as LFA-1 by "so called" inside out signalling (reviewed by Lub *et al* 1995). Activation may also cluster integrins on the cell surface and increase the avidity of binding. CD44 may play a role in binding MIP-1β, thus immobilising it for presentation to receptors on leucocytes (Tanaka *et al*

1993). E-selectin may also perform the dual function of tethering and activation of leucocytes (reviewed by Hogg, 1993).

Arrest of rolling then occurs by adhesion of the now functional integrins to the endothelium, via adhesion molecules of the immunoglobulin superfamily (Figure 3 C). ICAM-1 and 2 and VCAM-1 are the 3 major cutaneous endothelial ligands for integrins. Interaction of LFA-1 or MAC-1 to ICAM-1, VLA-4 and VCAM-1 or LFA-1 and ICAM-2 promotes strong attachment of leucocytes to the vessel wall. ICAM-1 and 2 are expressed constitutively at low levels on vascular endothelial cells. VCAM-1, E-selectin, ICAM-1 and ICAM-2 can be up-regulated by TNF-α and IL-1; whereas IL-4 induces V-CAM-1 but not ICAM-1 or E-selectin (Schleimer *et al*). Histamine and thrombin can also modulate adhesion molecule expression on endothelial cells (reviewed by Hogg, 1993).

The final stage is transendothelial migration from the blood vessels through the endothelial cell wall, surrounding pericytes and basement membrane and into the tissues (Figure 3 D). Once in the tissues, leucocytes move towards an increasing concentration of chemoattractant (chemotaxis). Alternatively haptotaxis may occur, where cells migrate towards the highest density of adhesive ligands (although this has only been demonstrated *in vitro*) (reviewed by Springer, 1994). The secretion of cytokines such as IL-1 IL-8, MCP-1, RANTES and IL-6 play a role in migration of leucocytes from blood.

3.1.2 Cutaneous trafficking

Components of the skin and its associated lymphoid system is shown in Chapter 1 section 1.1. KC are the most abundant cells in the epidermis and together with melanocytes comprise the majority of resident epidermal cells. Most other cell types found in the epidermis migrate from the blood into the skin, many remain in the dermis but some penetrate the basement membrane and enter the epidermis. LC

migrate into the epidermis from the blood where they remain until they are induced to migrate to the draining lymph node, to present antigen to naive T cells. The epidermis is then repopulated with LC derived from progenitors in the blood, although it is not known what regulates this process. Other APC such as macrophages migrate into the skin along with granulocytes but do not recirculate, whereas skin homing T cells migrate from the blood vessels into the skin and recirculate back into the blood via the afferent lymphatic and draining lymph node.

The endothelium of the post capillary venules can be induced by cytokines to express selectins and cell adhesion molecules thus promoting trans-endothelial migration (described in section 3.1.1). In resting skin, small numbers of lymphocytes can be observed around dermal blood vessels and in the basal layer of the epidermis (Foster *et al* 1990), suggesting that movement of cells into the skin is part of the normal immunosurveillance. In normal skin ICAM-1 is constitutively expressed on vascular endothelial cells, presumably facilitating movement of leucocytes into the dermis. There is evidence for preferential trafficking of leucocyte subsets to particular sites since priming of T cells by a specific antigen in a particular environment induces surface receptors that enables preferential recirculation to the site where antigen was first encountered (Mackay, 1992). ICAM-1 is upregulated on endothelial cells and induced on epidermal KC in psoriasis and other cutaneous inflammatory reactions (Dustin *et al* 1988; Singer *et al* 1989; McGregor *et al* 1992; Barker, 1991). E-selectin is not constitutively expressed in resting skin but is induced on the surface of activated endothelial cells in inflammation (Bevilacqua *et al* 1987). E-selectin was initially found to bind to a ligand on neutrophils (Bevilacqua *et al* 1989) and was later shown to bind a subset of memory T cells *in vitro* (Picker *et al* 1991). These cells are $CD4^+$ $CD45Ra^-$ $CD45RO^+$ $CD58^{high}$ and express cutaneous lymphocyte associated antigen (CLA). CLA^+ T cells are found in greater numbers in the skin than other non-lymphoid tissues and it is thought that the CLA-E-selectin interaction is a skin specific homing

pathway (Picker *et al* 1991; Berg *et al* 1991). CLA expression is dependent on TGF-β and is induced by IL-12 (Leung *et al* 1995).

The adhesion of L-selectin to the peripheral lymph node addressin (PNad) forms a homing pathway to the peripheral lymph node, since expression of PNad is restricted to high endothelial cells or similar endothelium. Endothelium in resting skin does not express this L-selectin ligand, but some inflammatory cutaneous lesions express vascular PNad when there is an extensive mononuclear cell infiltrate in the skin (Michie *et al* 1993). This suggests that the L-selectin-PNad mediated homing pathway is induced in skin, only after the initial influx of lymphocytes. The pathway may act only with intense or persistent stimuli to augment cellular recruitment by E-selectin and ICAM-1.

3.1.3 Trafficking towards the epidermis

ICAM-1$^+$ KC bind LFA-1$^+$ T cells *in vitro* and is a proposed mechanism for the epidermotropism of T cells *in vivo* (Dustin *et al* 1988; Nickoloff *et al* 1989). This finding is supported by the observation that intra-epidermal T cells are often localised adjacent to ICAM-1$^+$ KC (Morelli *et al* 1994). *In vivo* studies show a high correlation of LFA-1 expression on leucocytes with an ability to migrate to the epidermis in mice (Shiohara *et al* 1989). Anti-LFA-1 antibodies also inhibit the migration of T cells to the epidermis (Shiohara *et al* 1989) and the development of a DTH response in mice (Kondo *et al* 1994). Viac *et al* (1992b) provide evidence for an important role of ICAM-1 in the movement of T cells into the epithelium of HPV infections at mucosal sites.

3.1.4 Local changes in HPV infection

A decrease in the density of epidermal LC has been reported in non-regressing cutaneous (Viac *et al* 1992a; Chardonnet *et al* 1986; Chardonnet *et al* 1983) and mucosal warts (Viac *et al* 1992a; Hughes *et al* 1988; Tay *et al* 1987a). This

decrease of LC may contribute to the lack of an immune response and wart persistence in these patients to HPV since LC are the major antigen presenting cell of the skin. This idea is supported by the observation that warts which were regressing did not have a reduction in the epidermal LC density (Chardonnet *et al* 1986; Fierlbeck *et al* 1989).

Semi-quantitative studies of mononuclear cells in cutaneous warts have shown some dermal T cell infiltrate with no predominance of either T cell subset (Viac *et al* 1992a; Viac *et al* 1992b; Chardonnet *et al* 1983). When plane warts regress the mononuclear cell infiltrate in the epidermis is found adjacent to damaged KC (Iwatsuki *et al* 1986), thus supporting the role of CMI in clearing HPV infection. A T cell infiltrate has been observed in genital warts with the predominance of $CD8^+$ T cells in the epithelium (Tay *et al* 1987b) or in the whole lesion (Viac *et al* 1992b). This infiltrate may be due to changes in adhesion molecule expression within the HPV lesion, which could alter the trafficking of mononuclear cells to the wart. ICAM-1 has been observed in the vascular endothelium of laryngeal and cutaneous warts. However, epidermal expression was not observed in cutaneous warts (Viac *et al* 1992b).

3.2 Aim

The aim of this chapter is to investigate whether HPV infection modulates cutaneous immunity. First the numbers of infiltrating T cells (CD3) and memory T cell phenotype (CD45RO) in the dermis and epidermis, and number of LC in the epidermis of these lesions were quantified in cutaneous warts. In addition the areas of ICAM-1 and E-selectin expression were quantified in these lesions. The second approach was to investigate whether HPV modulates the function of adhesion molecule expression within the skin. A frozen section adhesion assay of activated T cells was undertaken as an *in vitro* model of lymphocyte homing (Stamper *et al*

1976). In view of the potential importance of the ICAM-1 and LFA-1 interaction, both for the movement of lymphocytes into the dermis from the blood vessels and from the dermis to the epidermis, adhesion was blocked using monoclonal antibodies to ICAM-1 and LFA-1. Comparisons were made throughout between sections of skin from normal subjects and involved skin of patients with chronic plaque psoriasis, a disorder in which the influx of T cells into the epidermis has been attributed to ICAM-1 and LFA-1 interaction (Barker *et al* 1992).

3.3 Materials and methods

3.3.1 Immunohistochemistry

3.3.1.1 Patients

Viral warts were obtained from 8 patients aged 17-55 years. Five were common facial warts which had been present for less than 1 year (numbers 1-5). The remaining three were a plantar wart (number 6) and two hand warts (numbers 7 and 8) all of which had persisted for at least two years. None of the warts had been treated prior to their removal by curettage. All specimens were bisected, half being formalin-fixed for routine histological examination, the remainder being snap frozen in liquid nitrogen and stored at -70°C. Control specimens consisted of 6 mm punch biopsies from normal forearm skin of six volunteers (age 20-57 years) and from involved skin of four psoriatic patients (age 21-46 years).

3.3.1.2 Immunoperoxidase staining of sections

Five μm cryostat sections were cut and air dried overnight before fixing in acetone for 20 min at room temperature. Slides were air dried and stored at -20 °C until used. Indirect immunoperoxidase staining was performed using the mouse anti-human monoclonal antibodies listed in Table 3.1.

Table 3.1 Primary antibodies used in immunohistochemistry

Antibody	Source	Product Code	Dilution
anti-CD3	DAKO	M 835	1 / 20
anti-CD1a	DAKO	M 721	1 / 20
anti-CD45R0	DAKO	M 742	1 / 200
anti-ICAM-1	R and D systems	BBA 3	1 / 200
anti-E-selectin	R and D systems	BBA 1	1 / 100

R and D systems (Abingdon, UK). DAKO (High Wycombe, UK)

Sections were immersed in tris-buffered saline (TBS) pH 7.6, and endogenous peroxidase inactivated with 0.6% hydrogen peroxide in ethanol for 5 min followed by blocking in 20% rabbit serum in TBS for 20 min. Sections were washed 3 X in TBS after each step. Primary antibodies diluted as shown in Table 3.1 in 10% human serum in TBS, were added to the slides and they were incubated in a humid chamber at 4°C overnight. The human serum used throughout was from the same pooled batch which had been heat inactivated. Ten % human serum in TBS was used as a control and additional controls contained irrelevant monoclonal antibodies of the same isotypes as those used in Table 3.1. The slides were washed in TBS and goat anti-mouse horseradish peroxidase conjugated antibody (DAKO), diluted 1/100 in 10% human serum in TBS, was layered on top of the slides which were incubated for 30 min at room temperature. The developing substrate was one 10 mg diaminobenzidine tetrahydrochloride tablet (Sigma) dissolved in 15 ml TBS containing 12 µl 30% hydrogen peroxide solution filtered through a 0.2 µm filter. Slides were lightly counterstained with haematoxylin to enable visualisation of the dermis and epidermis before dehydrating the slides through 30%, 70% and 100% ethanol and finally immersing in xylene and mounting in DPX.

3.3.1.3 Quantification of cell numbers and area of adhesion molecule expression

The stained areas were quantified using image analysis of sections and a semi-automated quantification method using the TPL software version 5 on the Seescan

image analysis system (Seescan Plc, Cambridge, UK). Areas of dermis and epidermis were traced on the video screen and numbers of positively stained cells and area of positive staining was calculated per μm^2 (or mm^2) dermis or epidermis, based on at least three fields of view at x 10 magnification (mean of three separate measurements) depending on the size of the section. Expression of adhesion molecules was calculated as area stained per area measured, since positively stained endothelial cell numbers were difficult to determine. $CD3^+$ and $CD45RO^+$ T cells were expressed as numbers of cells stained per mm^2 of dermis and epidermis measured. $CD1a^+$ LC were expressed as number of cells stained per mm^2 epidermis measured.

3.3.2 Adhesion assay

3.3.2.1 Activation of lymphocytes

Although resting T cells express LFA-1 they lack the NK1-L16 epitope essential for *in vitro* aggregation and spontaneous adhesion; indeed no adherence above basal levels with unstimulated lymphocytes is found (Barker *et al* 1992). Stimulation with phorbol myristate acetate (PMA), activation with phytohaemagglutinin (PHA) via the T cell receptor and addition of interleukin 2 (IL-2) increase levels of NK1-L16 and change the status of LFA-1 from an inactive to an active form (Figdor *et al* 1990). In this study, the experiments without blocking were done following the method of Barker *et al* (1992). Heparinised blood from normal donors was diluted 1:1 in RPMI-1640 (Gibco) containing 10 % foetal calf serum (FCS) and separated on lymphoprep™ (Nycomed). The purified PBMC were washed X 3 in RPMI-1640 /10% FCS with 200 mM L-glutamine (Gibco) and were dispensed into 24 well plates at 1 x 10^6 / ml in medium containing 50 ng / ml PMA (Sigma) and incubated for 30 min at 37^0C in 95% air / 5 % CO_2. To facilitate the measurement of any inhibition caused by the blocking antibodies, the adhesion of cells to the skin sections was increased by activating the PBMC in medium containing 50 ng/ml PMA and 1 µg / ml PHA (Sigma) for six days. The medium was replaced and

supplemented with recombinant human IL-2 (Genzyme), 20 units / ml, on days three and five of culture.

3.3.2.2 Phenotyping of activated lymphocytes

The phenotype of the stimulated PBMC cell population for the adhesion assay was performed using the following mouse anti-human monoclonal antibodies (DAKO). CD3 (T cell receptor), CD14 (monocyte marker), CD18 (LFA-1 β chain), CD25 (IL-2 R α chain) and CD56 (NK cell marker). A fluorescein isothiocyanate (FITC) conjugated sheep anti-mouse IgG $F(ab')_2$ fragment was used as a secondary antibody (Sigma), and the samples were analysed by flow cytometry on a Coulter XL using XL software version 1.5 (Coulter).

3.3.2.3 Patients

Persistent viral cutaneous warts were curetted from 6 patients (aged 18-57 years) who had received no treatment several weeks prior to curettage. Three were plantar mosaic warts, 2 were common hand warts, and one was from the supra-pubic region. All specimens were bisected, half being formalin fixed for histological confirmation of the diagnosis. The remainder was snap frozen in liquid nitrogen and stored at -70°C. Control specimens consisted of 6 mm punch biopsies from normal forearm skin of six volunteers (aged 20 -57 years) and from involved skin of six patients with psoriasis (aged 18 - 75 years). Two specimens were from the trunk, two from the leg and two from the forearm. Cryostat sections of the biopsies (8 μm) were cut on to uncoated slides and air dried at room temperature before storing at -20 °C. On the day of the assay, sections were thawed and fixed in 2% glutaraldehyde in PBS for 5 min followed by washing in RPMI (2 X 10 min).

3.3.2.4 Blocking sections with anti-ICAM-1

Fixed tissue sections were incubated for 15 min with 20% rabbit serum in TBS. Incubation for 1 hr at room temperature in 1/50 dilution of anti-ICAM-1 monoclonal

antibody (BBA-3, 1 mg / ml, R + D systems) in 20% rabbit serum in 0.1 M TBS pH 7.6 in a humid chamber. Control sections were incubated with diluent only. This dilution of antibody was shown to be at a saturating concentration for functional blocking by use of a range of dilutions in a preliminary study. Excess fluid was removed from the slides. The monoclonal antibody that remained was present throughout the subsequent adhesion assay.

3.3.2.5 Blocking PBMC with anti-LFA-1 antibody

Activated PBMC described in section 3.3.2.1 were blocked in TBS containing a 1/50 dilution of anti-CD18, the β 2 chain of LFA-1 (MHM 23, DAKO, 206 μg / ml) at room temperature for 1 hr. The cells were then used without washing so that some antibody was present throughout the adhesion period. Since adhesion mediated by integrins or selectins is an energy dependent process which requires divalent cations (Figdor *et al* 1990; Hogg, 1993), additional sections were blocked with adhesion buffer containing 5 mM EDTA or 0.01% sodium azide to determine if the adhesion was integrin / selectin mediated. The experiment with EDTA and sodium azide was performed on psoriasis sections only.

3.3.2.6 Adhesion

The adhesion method has been described previously (Nickoloff *et al* 1989; Barker *et al* 1992). Briefly, activated PBMC as prepared in section 3.3.2.1 were harvested and resuspended at 1.5 x 10^6 / ml in adhesion buffer. Adhesion buffer was minimum essential medium (MEM) (Gibco) containing 40 mM tricine buffer pH 7.4 (Sigma) and 1 mg/ml bovine serum albumin. 400 μl of blocked or untreated activated PBMC was layered carefully on top of the tissue sections and incubated for 45 min at 37°C with gentle agitation. Slides were then dipped X 5 in adhesion buffer to remove any non-adherent PBMC from the sections. Washed tissue sections were fixed in acetone for 20 min at room temperature, followed by blocking in 20% rabbit serum in TBS for 20 min. $CD3^+$ and $CD18^+$ leucocytes were stained using

immunoperoxidase as described in section 3.3.1.2 using 1/20 dilutions of mouse anti-human CD3 (DAKO) or anti-human CD18 (DAKO).

3.3.2.7 Quantification of adherent cells

$CD3^+$ or $CD18^+$ adherent T cells could be easily distinguished from endogenous T cells as they were above the plane of the tissue section. Numbers of adherent cells were counted per mm^2 of normal skin (N), warts (W), and psoriasis skin (P) using image analysis described in section 3.3.1.3. Each experiment consisted of the lymphocytes from one individual used to adhere to sections of at least 2 W, 2 N and 2 P (from a total of 6 for each skin type), and different blood donors were used in each experiment. The adhesion was expressed as means of number of adherent cells / mm^2 of dermis and epidermis or as a percent inhibition of binding. The error bars are the standard error of the means between experiments. Percentage inhibition was calculated by :

$$100 - \left(\frac{\text{number of cells bound / mm}^2\text{ tissue with antibody present}}{\text{number of cells bound / mm}^2\text{ tissue}} \times 100 \right)$$

3.3.3 Statistical analysis

Standard deviation from the means was calculated in all cases and statistical analysis was performed using the Mann-Whitney U test using Systat for the Macintosh version 5.2.1

3.4 Results

3.4.1 Immunohistochemistry

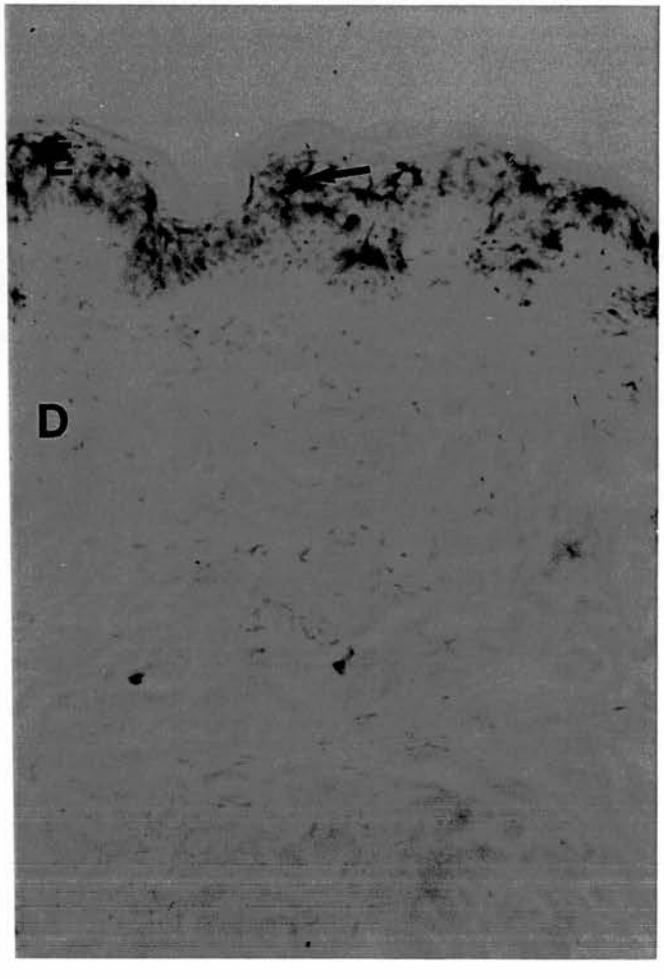
Wart, normal skin and psoriasis sections were stained with the panel of monoclonal antibodies (Table 3.1) or isotype matched controls. Photomicrographs of representative tissue sections are illustrated, and the quantification of the positively staining cells and areas of staining are shown. Non-specific staining was not

observed in any of the controls which were incubated with either diluent or isotype matched controls.

3.4.1.1 Langerhans' cells

CD1a staining of LC in 1 normal skin sample and 2 cutaneous wart specimens are shown below.

Figure 3.2 CD1a+ LC in normal skin



Photomicrograph of normal skin stained with CD1a antibody. D = dermis, E = epidermis. Magnification = X 50. The arrow indicates a LC with typical dendritic morphology.

Figure 3.3 CD1a+ LC (high) in a cutaneous wart

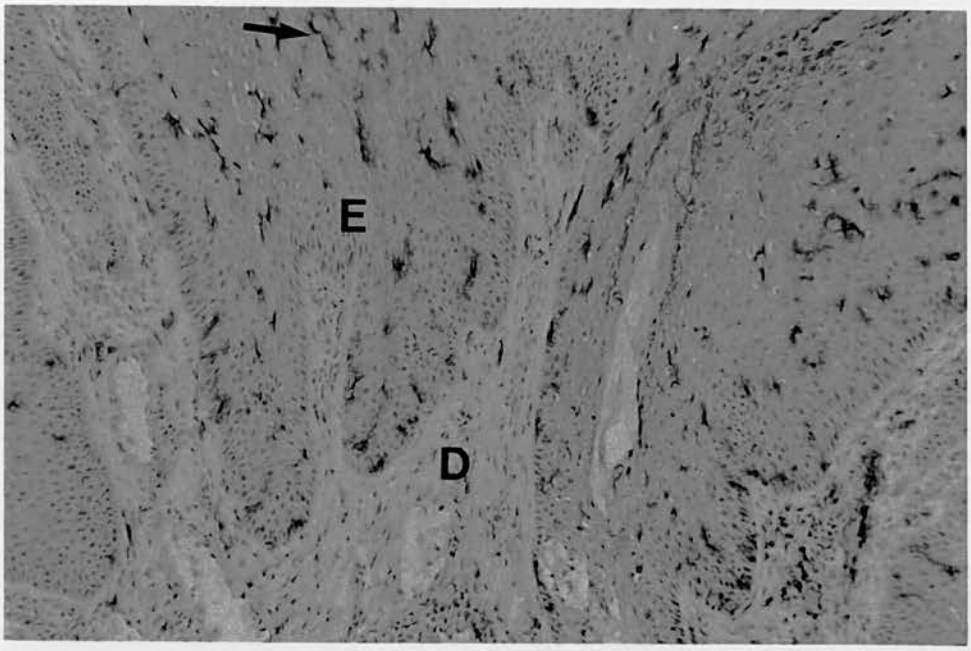
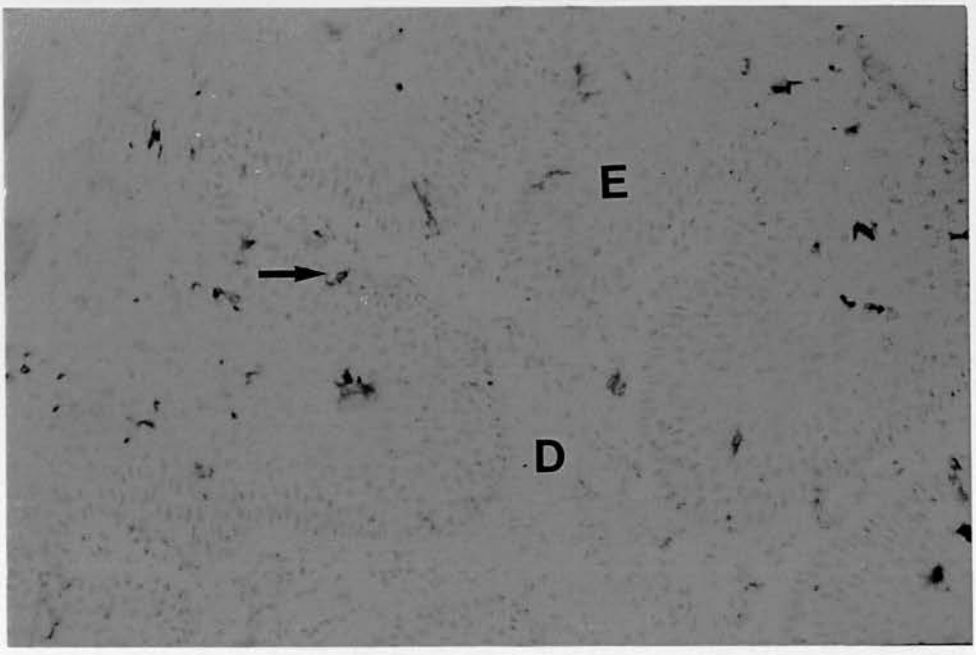


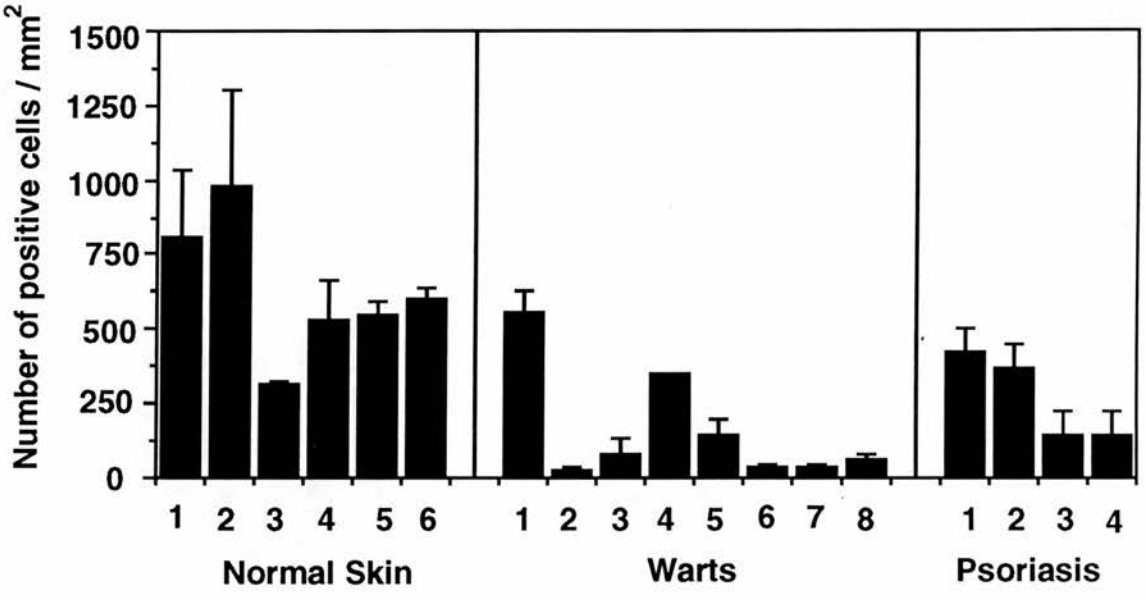
Figure 3.4 CD1a+ LC (low) in a cutaneous wart



Photomicrographs of high numbers of CD1a $^{+}$ LC in a wart (Figure 3.3) and low numbers of CD1a $^{+}$ LC in a wart (Figure 3.4). Magnification = X 50. D = dermis, E = epidermis. The arrow on Figure 3.3 indicates LC throughout the depth of the epidermis The arrow on Figure 3.4 indicates CD1a^{+} LC in the basal layers of the epidermis only.

CD1a$^+$ cells with dendritic morphology typical of LC were strongly expressed in the epidermis of normal skin (Figure 3.2). Figure 3.5 shows quantification of LC density in the epidermis. of the 3 skin types.

Figure 3.5 Quantification of LC numbers in the epidermis



Density of CD1a$^+$ LC expressed as numbers of cells stained per total area of epidermis measured (mm^2) in the 6 normal subjects, 8 with warts and 4 with psoriasis. Wart patients 1-5 had papilliferous lesions, patient 6 had plantar warts and 7 + 8 had common hand warts. Bars depict standard deviation from the mean. Numbers of CD1a$^+$ LC were significantly greater in the epidermis of normal skin compared with wart epidermis or with psoriatic skin ($p < 0.05$ in both cases). No significant difference was observed between the numbers of LC in the psoriatic epidermis and wart epidermis.

Intensely stained LC were observed throughout the depth of the epidermis in normal skin. The number of positive cells ranged from 314 ± 8 to 981 ± 318 per mm^2 epidermis. In contrast the warts had variable numbers of CD1a$^+$ LC. Warts 1 and 4 showed CD1a expression with a wide distribution throughout the epidermis (Figure 3.4). Staining was also observed in the papillary dermis although these cells had a

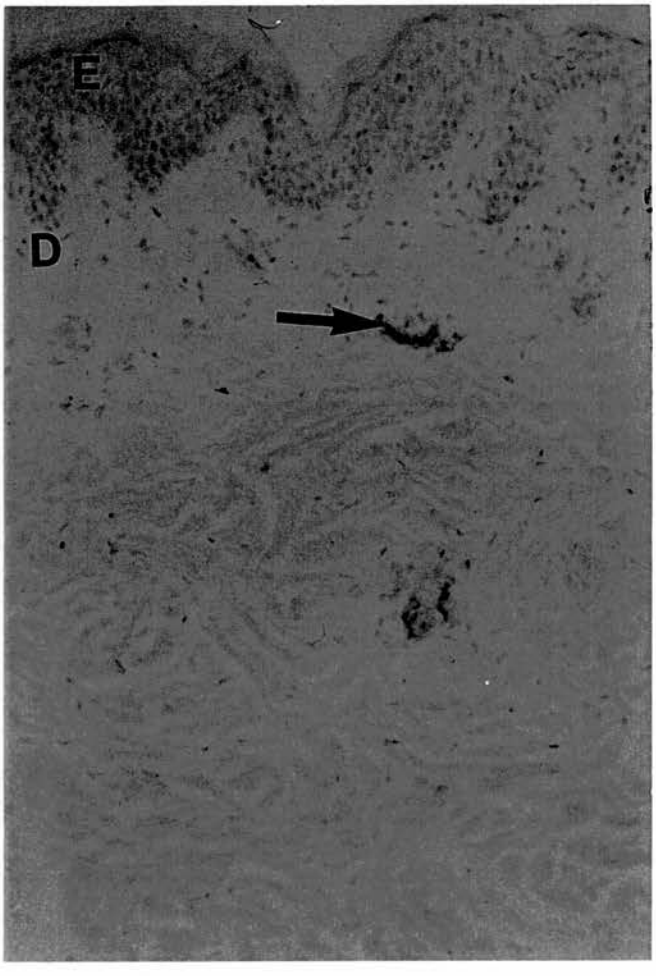
rounded morphology, and some CD1a$^+$ cells with dendritic morphology were seen around the blood vessels in the dermis. Warts 3 and 5 had a reduced density of CD1a $^+$ LC, although they were still evenly distributed throughout the epidermis (Figure 3.4). The other four warts had a few LC in the epidermis with CD1a expression only in the basal and mid-epidermis, and none in the dermis. The LC density in the epidermis of all 8 warts was significantly less than LC density in the epidermis of normal skin ($p < 0.05$). CD1a expression in the epidermis of psoriatic lesions was also significantly decreased in comparison with normal epidermis ($p = 0.05$) (Figure 3.5). No significant difference was observed between the CD1a expression in the epidermis of patients with warts and with psoriasis.

3.4.1.2 Adhesion molecule expression

ICAM-1 and E-selectin showed a similar vascular pattern of expression. The expression of E-selectin in normal skin, a cutaneous wart and ICAM-1 in a cutaneous wart are shown in Figures 3.6-3.8.

Figure 3.7 and 3.8 demonstrates that there are increased areas of ICAM-1 and E-selectin expression in the dermis of warts compared with normal skin. Patterns of expression were similar for these adhesion molecules in all sections. No epidermal ICAM-1 was observed. The areas of ICAM-1 and E-selectin expression in the dermis of normal skin, warts and psoriasis were quantified and is shown in Figure 3.9.

Figure 3.6 E-selectin expression in normal skin



Photomicrograph of normal skin stained with anti-E-selectin. D = dermis, E = epidermis. Magnification = X 50. Arrow indicates longitudinal section of vascular endothelial cells positively stained for E-selectin.

Figure 3.7 E-selectin expression in a cutaneous wart

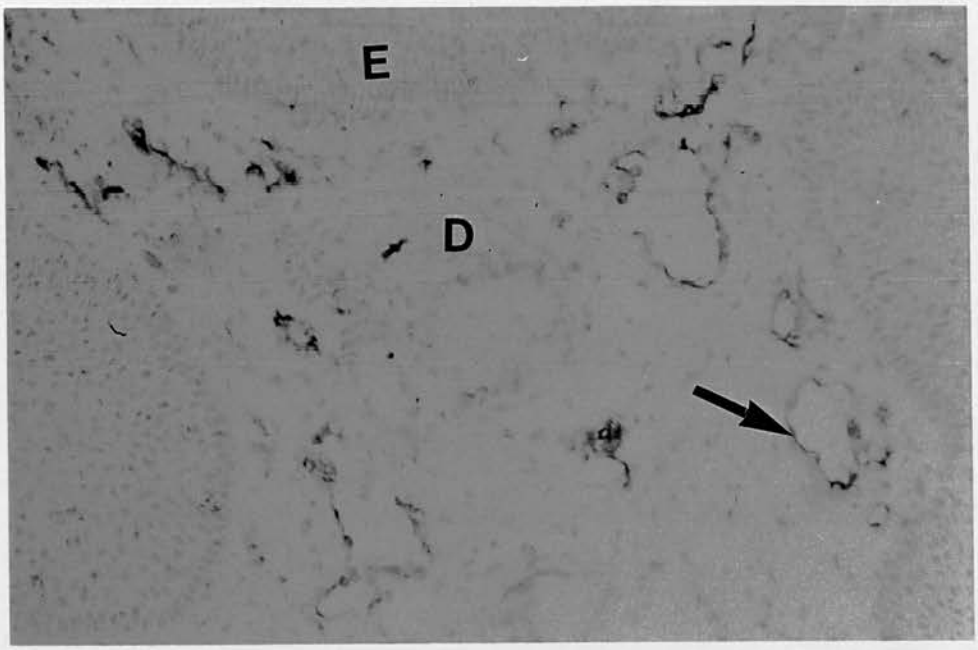
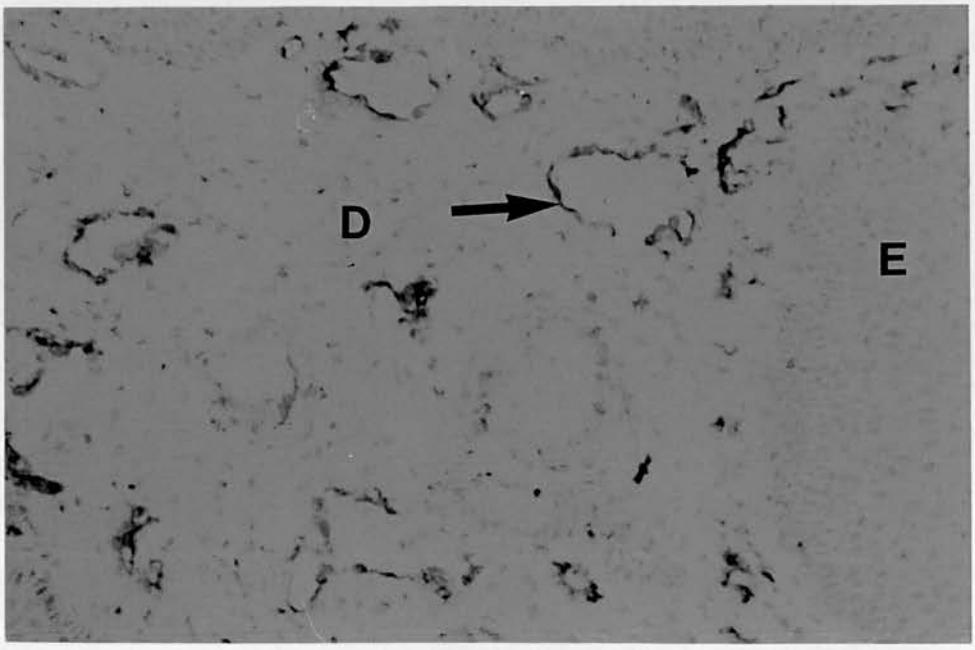
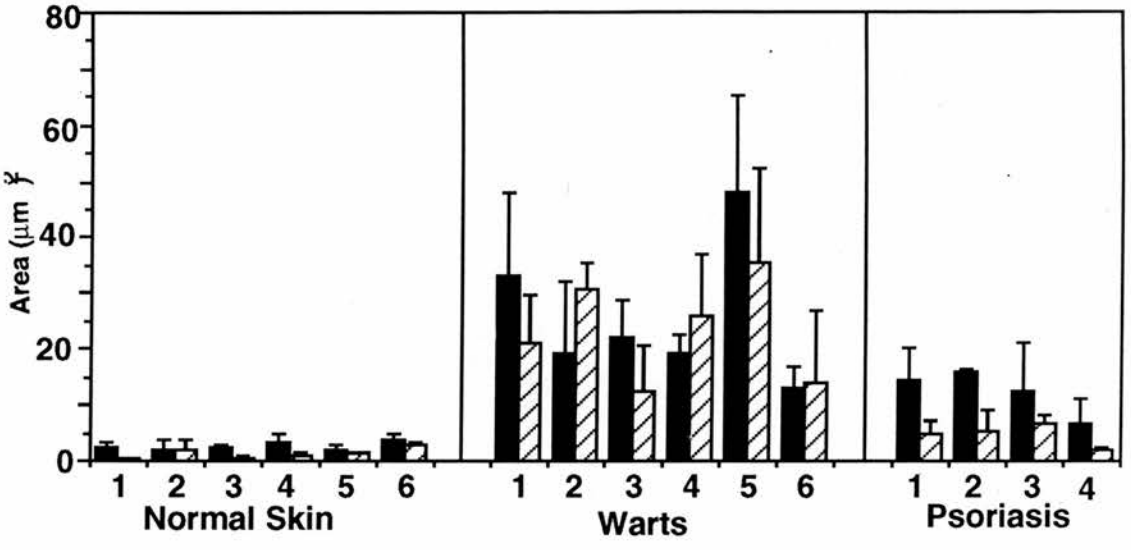


Figure 3.8 ICAM-1 expression in a cutaneous wart



Photomicrographs of stained sections at a magnification of X 50 show E-selectin expression and ICAM-1 expression in the same wart specimen (number 1). D = dermis, E = epidermis. The arrows in Figure 3.7 and 3.8 indicate E-selectin and ICAM-1 expression respectively, on vascular endothelium, in the highly vascularised wart dermis. No ICAM-1 positivity in the epidermis was detected.

Figure 3.9 Quantification of vascular adhesion molecule expression



Expression of adhesion molecules in the dermis of 6 normal subjects, 6 with warts and 4 with psoriasis. Positive staining is expressed as area stained per total area measured (μm^2 and bars depict standard deviation from the mean. ■ = ICAM-1, ▨ = E-selectin. The area of ICAM-1 and E-selectin staining in the dermis was significantly increased ($p < 0.005$ in both cases) in wart dermis compared with the expression in the dermis of normal skin. The ICAM-1 and E-selectin staining was also significantly greater in the dermis of psoriatic skin compared with normal dermis ($p < 0.05$ in both cases). No significant difference was observed in the expression of these adhesion molecules in the dermis of warts and psoriatic skin.

Normal skin had weakly stained areas of ICAM-1 and E-selectin on vascular endothelial cells (Figure 3.6) which was not observed elsewhere (with a range of 2 ± 1 μm^2 to 4 ± 1 μm^2 for ICAM-1 and 0 to 3 ± 0 μm^2 for E-selectin). In contrast, warts showed a significant increase in the areas of ICAM-1 and E-selectin stained (Figure 3.7 and 3.8). The areas stained ranged from 13 ± 4 μm^2 to 48 ± 17 μm^2 for ICAM-1 and 12 ± 8 μm^2 to 35 ± 17 μm^2 for E-selectin. Positive staining for both ICAM-1 and E-selectin was in longitudinal or circular strands typical of vascular endothelium in the dermis. The warts were more highly vascularized than the

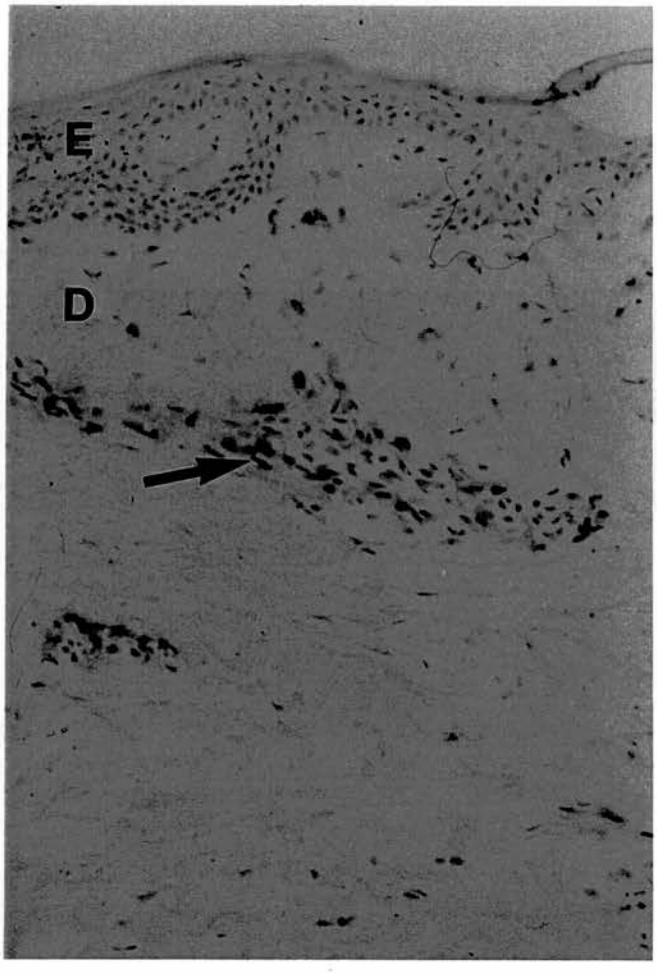
normal skin, and a high proportion of the vascular endothelial cells were positive in the warts. The dermis of psoriatic lesions also expressed ICAM-1 and E-selectin on the vascular endothelium as previously reported (Singer *et al* 1989; McGregor *et al* 1992; Barker, 1991; Scheynius *et al* 1992). Areas of adhesion molecule expression were significantly greater in the dermis of warts than in normal skin. No difference was seen between the expression of adhesion molecules in the wart dermis and psoriatic dermis. ICAM-1 positive KC were not detected in the epidermis of any sections tested in this study although ICAM-1 positive KC were detected in the epidermis of one patient with eczema (not shown).

3.4.1.3 T cells

The pan T cell marker (CD3) and the memory T cell marker (CD45RO) showed similar staining patterns, therefore only $CD3^+$ T cells are shown in normal skin, warts and psoriasis skin. Figure 3.10 -3.12 show $CD3^+$ T cells in the dermis and epidermis in warts, normal skin and psoriatic skin (respectively) and the quantification of these numbers are shown in Figure 3.13.

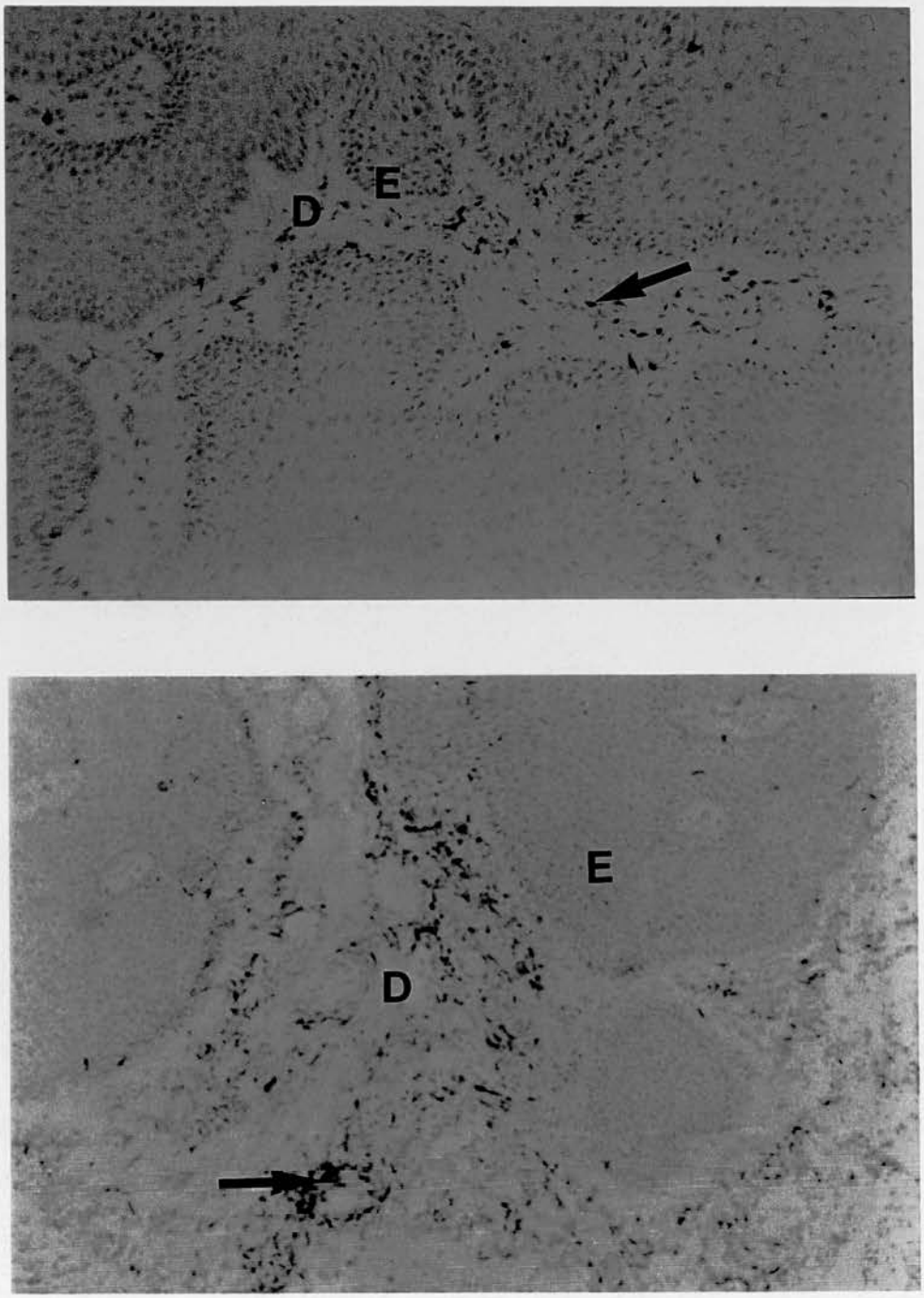
Normal skin had little or no mononuclear cell infiltrate into the dermis or epidermis. $CD3^+$ T cells in the dermis were in the range 26 ± 6 to 140 ± 88 per mm^2 and were not significantly different from the numbers found in the epidermis (0 to 166 ± 95 per mm^2). Cells of CD45RO phenotype were present at similar frequencies to the $CD3^+$ T cells in both the dermis and the epidermis, suggesting that most of the T cells present were of the memory phenotype (Figure 3.13). Both cell phenotypes were observed in the perivascular area of the dermis. The $CD3^+$ infiltrate varied in the wart patients from a mild (53 ± 36 cells / mm^2) to a strong dermal infiltrate (654 ± 242 cells / mm^2).

Figure 3.10 CD3 + T cells in normal skin



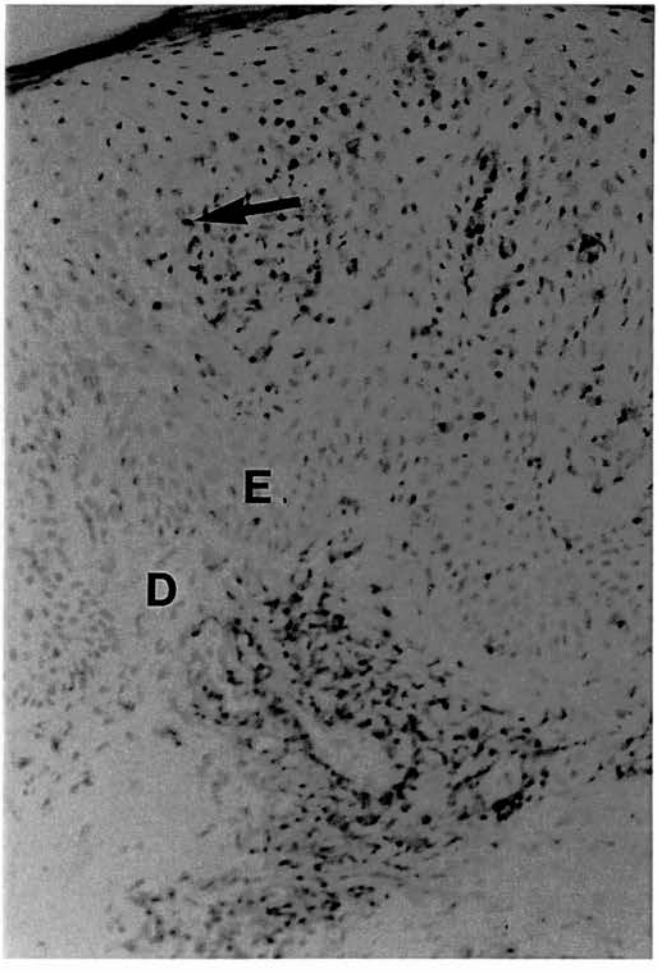
Photomicrograph of $CD3^+$ T cells in normal skin. magnification = X 50. D = dermis, E = epidermis. The arrow indicates positive T cells in the dermis of the normal skin.

Figure 3.11 CD3 + T cells in cutaneous warts



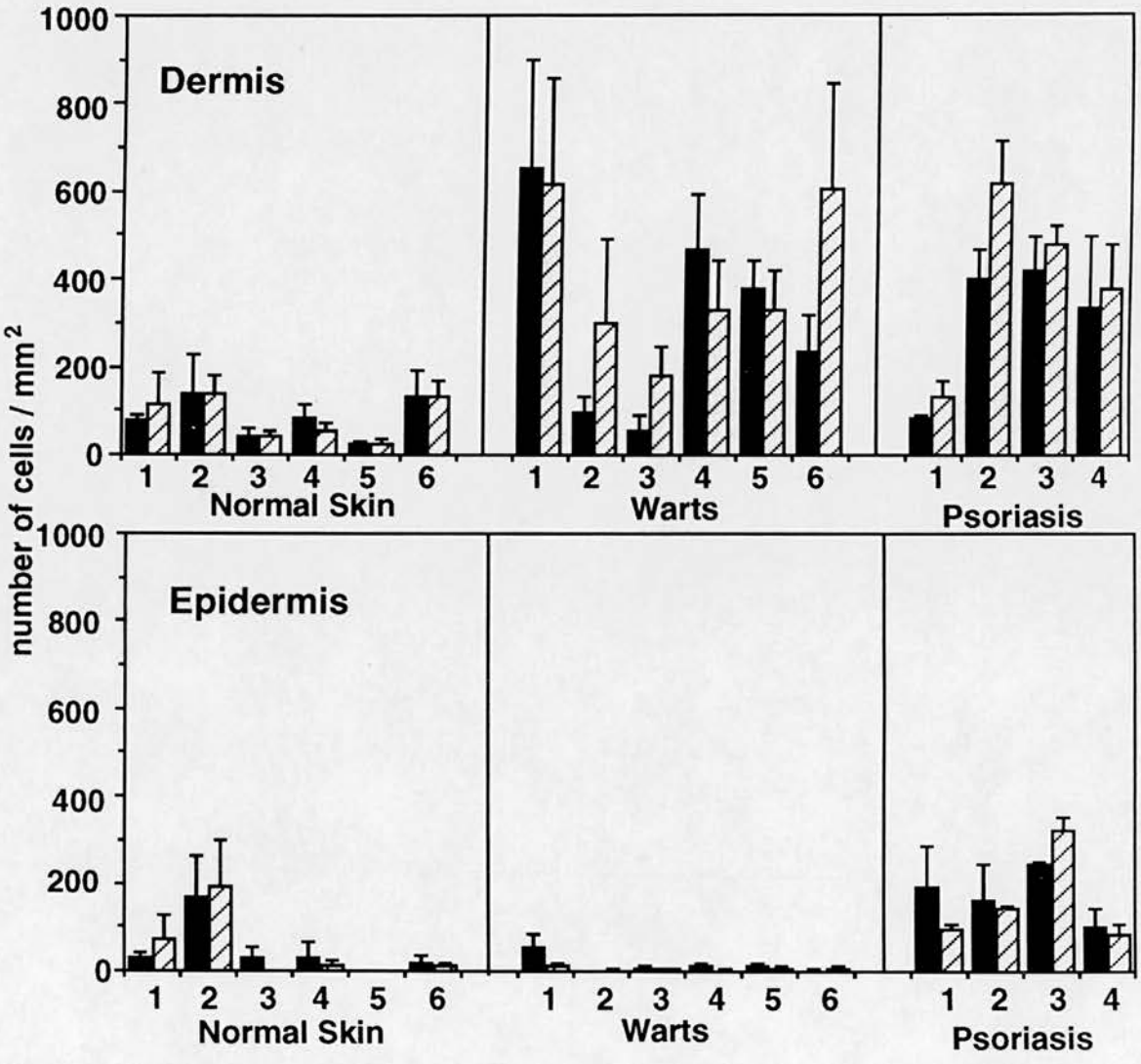
Photomicrograph of $CD3^+$ T cells in 2 cutaneous warts magnification = X 50. D = dermis, E = epidermis. The arrow indicates T cells in the dermis .

Figure 3.12 CD3+ T cells in psoriasis.



Photomicrograph of $CD3^+$ T cells in psoriasis. magnification = X 50. D = dermis, E = epidermis. The arrow indicates positive T cells in the epidermis.

Figure 3.13 Quantification of CD3+ and CD45 RO+ T cells



Numbers of T cells in the dermis and epidermis were quantified in 6 normal subjects, 6 with warts and 4 with psoriasis. $CD3^+$ = ■ and $CD45RO^+$ = ▨. Significantly greater numbers of T cells ($CD45RO^+$ and $CD3^+$ cells) were seen in the dermis of warts compared with normal dermis ($p < 0.05$ in both cases). No significant difference in the numbers of T cells was seen in the epidermis of warts and normal skin. T cell numbers were significantly greater in psoriatic epidermis as compared with wart epidermis ($p < 0.05$ for both $CD3^+$ and CD45 RO^+ cells).

Many of the infiltrating T cells were CD45 R0 positive (Figure 3.14). Interestingly, the numbers of $CD3^+$ and $CD45RO^+$ T cells decreased significantly in the epidermis of wart patients compared with the dermis ($p < 0.005$). Psoriatic lesions had comparable numbers of T cells infiltrating the dermis as the warts but more T cells in the epidermis. The wart epidermis was more comparable to normal epidermis in terms of intraepithelial T cell numbers.

3.4.2 Adhesion assay

3.4.2.1 Adherence of activated T cells to tissue sections

In studies without blocking, whole PBMC were stimulated for 30 min with PMA, and prior to their use in the adherence assay. This population of cells was 62% $CD3^+$ and 67% $CD18^+$ (Table 3.2).

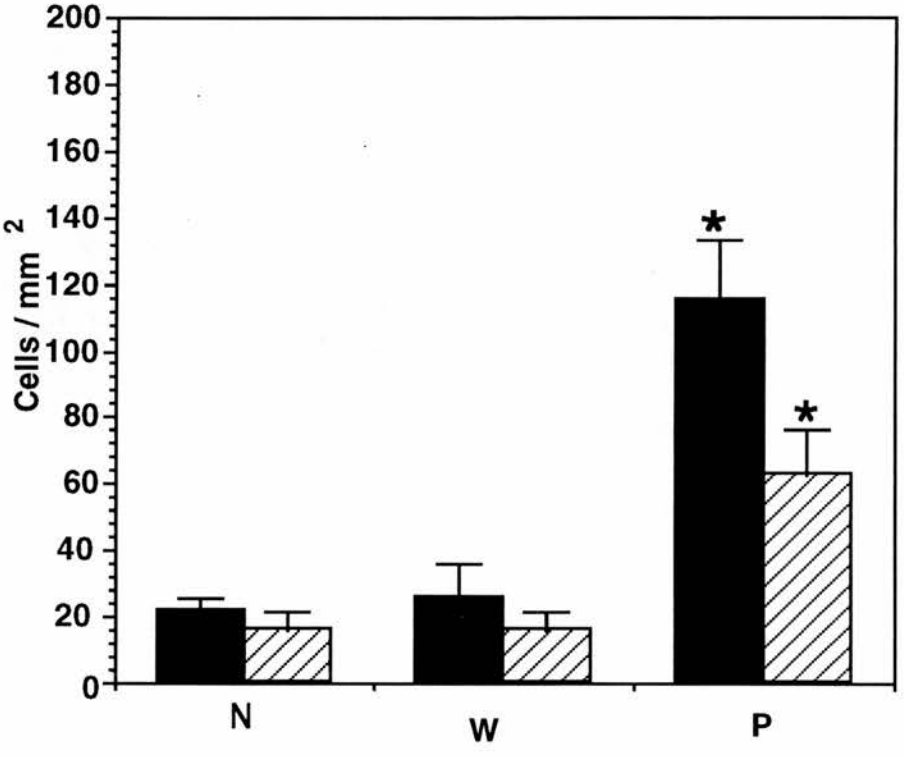
Table 3.2 Phenotype of the activated PBMC used in the adherence assay

	Cells used in studies without blocking	Cells used in studies with blocking
	30 min activation	Activated for 1 week
CD3	62 (+/- 8)	71 (+/- 8)
CD14	13 (+/-4)	4 (+/-1)
CD18	67 (+/- 4)	95 (+/- 1)
CD25	5 (+/-2)	86 (+/-3)
CD56	7 (+/- 2)	8 (+/- 3)

Values are mean percentages of positive cells from 5 individuals used in experiments without blocking and from three individuals used in experiments with blocking +/- standard error of the mean.

Adherence of $CD3^+$ T cells and $CD18^+$ cells to the epidermis and dermis of cutaneous warts, normal skin and psoriatic skin are shown in Figure 3.14 and 3.15. The adhesion of both these cell types to wart dermis and epidermis was significantly lower than the adhesion to the psoriasis dermis and epidermis ($p < 0.05$).

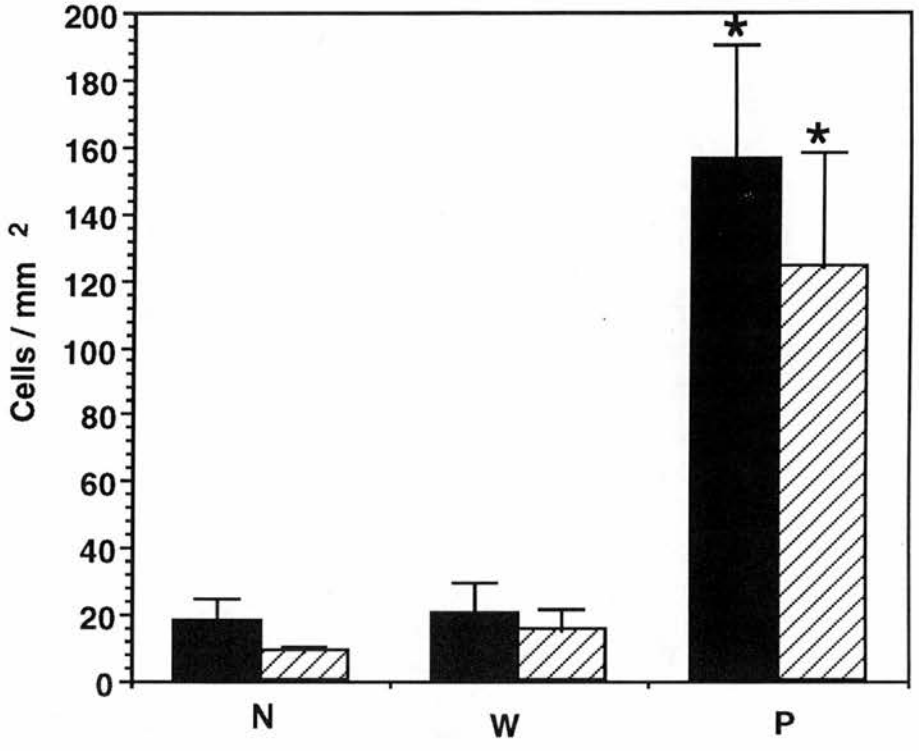
Figure 3.14 Adhesion of $CD3^+$ T cells to wart, normal skin and psoriasis skin



The adhesion of $CD3^+$ T cells to skin sections of normal skin (N), warts (W), and psoriasis skin (P). ■ = epidermis, ▨ = dermis. * = significantly different as determined by the Mann-Whitney U test ($p < 0.05$). SEM are from the means of 12 experiments each using 2 W, 2 P and 2 N sections.

The adherence of $CD3^+$ T cells to wart dermis and epidermis was 16 and 26 cells / mm^2 respectively, these numbers were similar to the adhesion of $CD3^+$ T cells to normal skin (16 cells / mm^2 for the dermis and 22 cells / mm^2 for the epidermis).

Figure 3.15 Adhesion of CD18+ T cells to wart, normal skin and psoriasis skin



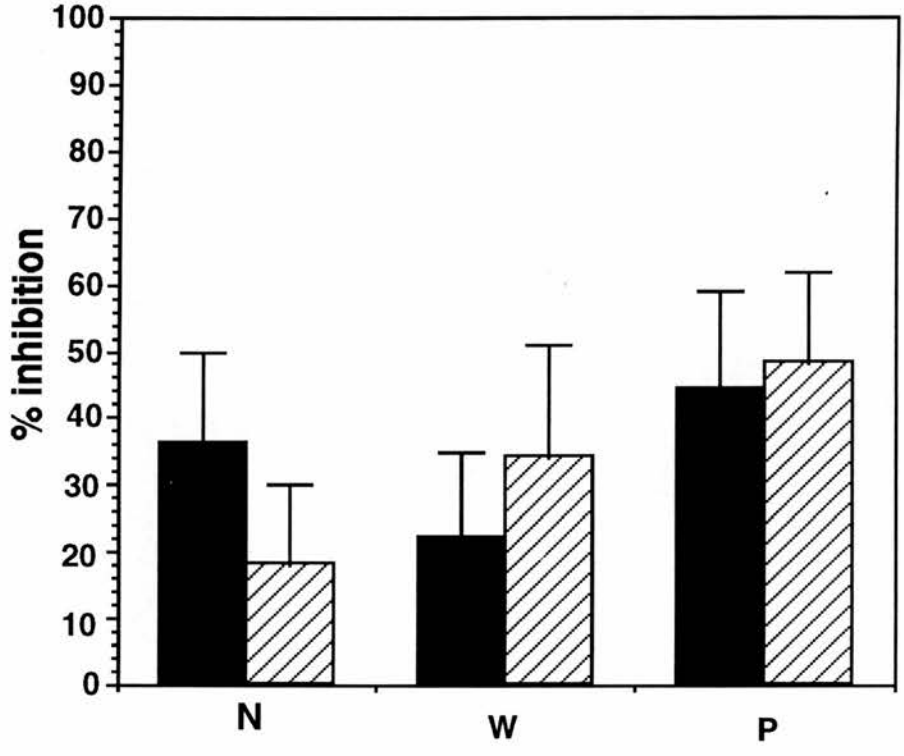
The adhesion of CD18+ cells to sections of normal skin (N), warts (W), and psoriasis skin (P). ■ = epidermis, ▨ = dermis. * = significantly different as determined by the Mann-Whitney U test ($p < 0.05$). SEM are from the means of 3 experiments each using 2 W, 2 P and 2 N sections.

Adhesion of CD18+ cells to tissue sections (Figure 3.15) showed a similar pattern of adhesion as CD3+ T cells. In both adherence assays there was no significant difference in numbers of cells adhering to normal skin dermis and epidermis compared with wart dermis and epidermis. The adhesion of CD3+ T cells to psoriatic dermis and epidermis was significantly increased compared with normal and wart dermis and epidermis, and confirms the work of (Barker *et al* 1992). There was also significantly greater adhesion of CD18+ cells to psoriasis skin compared with either normal skin or warts.

3.4.2.2 Blocking studies

For the blocking studies, the PBMC were activated for 1 week with PHA, PMA and recombinant human IL-2. The majority of these were $CD3^+$ (71%), while the percentage of $CD18^+$ cells in this population was 95% (Table 3.14).

Figure 3.16 Blocking CD3+ adhesion with anti-ICAM-1 antibody

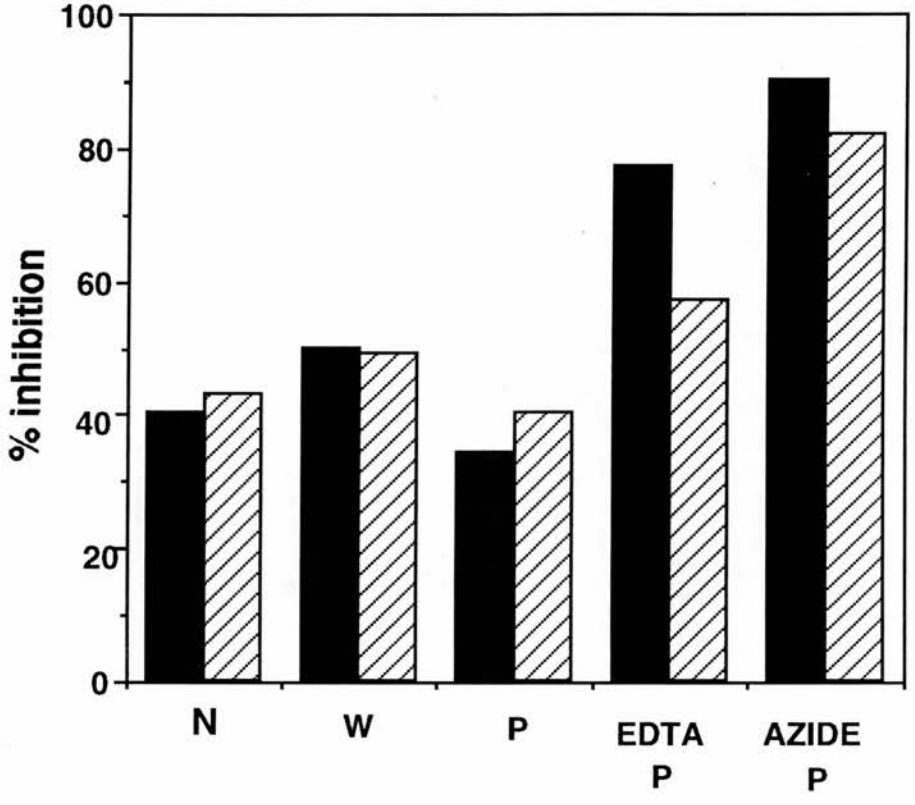


Blocking the adhesion of $CD3^+$ T cells with anti-ICAM-1 monoclonal antibody to sections of normal skin (N), warts (W), and psoriasis skin (P). ■ = epidermis, ▨ = dermis. SEM are from the means of 7 experiments.

Adhesion of $CD3^+$ T cells to normal skin, wart and psoriatic dermis and epidermis was partially blocked by anti-ICAM-1 monoclonal antibody, but total blocking was not achieved (Figure 3.16). Adherence to psoriasis skin was blocked to the greatest extent with a mean percentage inhibition of 44% and 48% for the epidermis and dermis respectively. The binding of $CD3^+$ T cells to wart dermis and epidermis was

inhibited by 34% and 22% respectively and the binding to normal skin epidermis and dermis was inhibited by 36% 18% respectively.

Figure 3.17 Blocking CD3+ adhesion with anti-LFA-1 antibody



The percentage inhibition of binding of $CD3^+$ T cells to normal skin (N), warts (W), and psoriasis skin (P) after using MHM23 LFA-1 monoclonal antibody. ■ = epidermis, ▨ = dermis. EDTA and AZIDE show the % inhibition of binding to psoriasis skin with 5 mM EDTA and 0.01% sodium azide added during the adhesion. No error bars are shown as the means from 2 experiments are shown.

Figures 3.17 shows the means of percentage inhibition when stimulated PBMC were blocked with MHM23 anti-LFA-1 monoclonal antibody prior to the adhesion assay. As with ICAM-1 blocking, only partial inhibition of binding of $CD3^+$ T cells to all three tissue types was achieved using MHM23. In addition the adhesion of activated

T cells to all 3 skin types was inhibited partially by the LFA-1 α chain antibody (WAC70, kindly supplied by Dr. J. Ross. Dept surgery, Royal Infirmary, Edinburgh) although this inhibition was never above 50%. Adhesion of activated T cells to psoriasis skin was inhibited by EDTA and sodium azide (Figure 3.17), indicating that the adhesion in this assay was dependent on divalent cations and was an energy dependent process. The percentage inhibition of binding in the presence of EDTA was 77% and 55% for the epidermis and dermis respectively. Sodium azide inhibited the adhesion to the epidermis and dermis by 90 and 82% respectively.

3.5 Discussion

3.5.1 Immunohistochemistry

3.5.1.1 Adhesion molecules and T cells

The increase in expression of ICAM-1 and E-selectin found in the dermis of warts can be partly accounted for by the increase in vascularization of these lesions. Although the intensity of staining was not included as part of the quantitative analysis, there was a greater intensity of ICAM-1 and E-selectin expression on endothelium in warts and psoriasis skin compared with normal skin.

It has been suggested that E-selectin plays a role in initiating the adhesion of resting T cells and neutrophils to activated endothelium. The CLA marker on T cells has been proposed as the ligand for E-selectin (Picker *et al* 1991), and this interaction may represent a skin-specific homing mechanism, for the movement of memory T cells to cutaneous sites. The results presented in this study suggest that E-selectin may be important in the trafficking of memory T lymphocytes through vascular endothelium to the wart dermis.

Upregulation of integrins on the T cell surface promotes strong adhesion to the endothelium via VLA-4-VCAM-1, LFA-1-ICAM-2 and LFA-1-ICAM-1 interactions, and leads to extravasation into surrounding tissues. Only the expression of ICAM-1 has been considered here although ICAM-2 was expressed on the vascular endothelium of wart dermis (data not shown). The expression of ICAM-1 at high levels in the dermis of warts demonstrates that this adhesion molecule also has a role in the homing of T cells into these lesions. E-selectin and ICAM-1 were both expressed at low levels in the dermis of normal skin, which may indicate that they play a role in the normal surveillance of cutaneous sites.

Studies on cervical intraepithelial neoplasia have also shown the vascular expression of ICAM-1, E-selectin and VCAM-1 (Coleman *et al* 1994). Increasing areas and intensity of adhesion molecule expression were found in samples exhibiting more severe grades of dysplasia.

ICAM-1 expression was not detected in epidermal KC in cutaneous warts, in agreement with results of other studies (Viac *et al* 1992a). ICAM-1^+ KC have been seen in some vulval condylomata (Morelli *et al* 1994), high grade cervical intraepithelial neoplasia and genital warts (Coleman *et al* 1994). ICAM-1 is also induced on the epidermal KC of regressing genital warts (Coleman *et al* 1994). Weak and scattered foci of epithelial ICAM-1 staining were found in HPV lesions which were low grade or without dysplasia. These findings may suggest a different host response at cutaneous sites compared with mucosal sites of HPV infection. Alternatively, changes in ICAM-1 expression on KC may be associated with the transformation of the cell rather than HPV infection alone. Indeed, Coleman *et al* (1993) observed constitutive ICAM-1 expression only in fully transformed tumourigenic cell lines which contained HPV, *in vitro*. In contrast, an HPV-16 infected cell line which retained episomal DNA copies did not constitutively express ICAM-1.

Studies on regressing genital warts have shown intense ICAM-1 and MHC class II KC expression, together with a significantly increased epithelial infiltrate of $CD4^+$, $CD8^+$ and $CD25^+$ T cells (Coleman *et al* 1994). In the epithelium, the number of $CD4^+$ T cells was greater than $CD8^+$ cells in regressing lesions, whereas $CD8^+$ cells were predominant in non-regressing warts.

ICAM-1 has been detected in the KC of laryngeal HPV-induced lesions only when intensely infiltrated with mononuclear cells (Viac *et al* 1992b). This could provide an explanation of why there are low numbers of T cells in the epidermis of warts, since interaction between ICAM-1^+ KC and LFA-1^+ T cells may have a role in the epidermotropism of T cells. However, epidermal KC in psoriatic lesions were also negative for ICAM-1 in this study, despite the presence of many intraepithelial T cells. Thus an ICAM-1 independent mechanism for this movement into the epidermis may be operating. ICAM-1 has been demonstrated on the KC of psoriatic lesions (Singer *et al* 1989; McGregor *et al* 1992; Barker, 1991) although Scheynius *et al* (1992) found expression on only 45% of involved psoriatic lesions. This suggests that ICAM-1 may not be the sole pathway of epidermal movement, and may only be induced after an intense infiltrate has entered the epidermis.

It has been postulated that LC may have a role in epidermotropism of T cells in mice (Shiohara *et al* 1988), however this was based on the observation that increased numbers of LC in the epidermis correlated with an influx of T cells. Indeed, in one study, normal skin was found to contain epidermal T cells which were not localised next to ICAM-1^+ KC (Konter *et al* 1989). Treatment of KC with γ-IFN induces the expression of ICAM-1 (Dustin *et al* 1988; Barker *et al* 1989) and therefore infiltrating T_H1 cells may be the inducer of epidermal ICAM-1 expression. However, studies in mice showed that intradermal injection of epidermotropic T cells caused intense epidermal ICAM-1 expression before the T cells had entered the epidermis (Shiohara *et al* 1989). High levels of ICAM-1 expression on KC is

observed in drug induced cutaneous vasculitis, where the infiltrating cells remain perivascularly. This may indicate that activated T cells in the epidermis are not necessary for ICAM-1 induction on KC, but activated T cells in the dermis may be sufficient to induce this adhesion molecule. In addition there is some evidence that contact allergens may induce ICAM-1 directly (Villada *et al* 1992; Griffiths *et al* 1989).

3.5.1.2 Langerhans' cells

The present study showed variable numbers of LC in the epidermis of HPV-induced warts which is in agreement with previous findings (Chardonnet *et al* 1986; Chardonnet *et al* 1983). Seventy five percent (6/8) of warts showed greatly reduced LC numbers while the remainder showed numbers comparable with those seen in the normal epidermis. LC in the warts with reduced numbers of CD1a staining were located towards the basal layer of the epidermis. This irregular distribution of LC is also found in vulval condyloma (Morelli *et al* 1994). Since nothing is known about the migration of LC to and from the epidermis into the draining lymph node during a chronic infection such as with HPV this finding remains difficult to explain. There was no clinical or histopathological evidence that the warts which showed no reduction in LC density were regressing. The two warts without a reduction in LC density were facial lesions, but other warts from the same clinical group also had decreased LC numbers. An increase in LC numbers has been observed in regressing cutaneous warts and it has been suggested that LC play a role in this regression (Chardonnet *et al* 1983; Iwatsuki *et al* 1986; Thivolet *et al* 1982). However, a significant increase in LC numbers was not observed in regressing genital warts (Coleman *et al* 1994). Regressing plane warts have a high LC density in the dermis and epidermis with T cells located adjacent to LC, and lymphocytes next to damaged KC in the epidermis (Iwatsuki *et al* 1986) which suggests a specific cell mediated immunity against viral infected KC.

Some reports indicate that there is an increase in LC numbers in the epidermis of non-viral skin tumours (Thivolet *et al* 1982), suggesting that HPV infection may contribute to the observed reduction in LC density in warts. However, other studies found a decrease in LC density in these tumours (Acalay *et al* 1989; Chen *et al* 1989). Chardonnet *et al* (1986), analysed a large number of cutaneous warts from different anatomical sites and found a reduction in LC densities in most but not all warts. There was some indication that the location of the wart could influence the number of LC since 47% of plantar warts, 29% of hand warts and only 14% of genital lesions had no epidermal LC (Chardonnet *et al* 1986). However, this may not be due to HPV infection since one study found no differences in LC density at different anatomical locations with the exception of the soles of the feet which had significantly less LC in the epidermis (Berman *et al* 1983). It has been demonstrated previously that the depletion of LC in the epidermis correlated with the presence of HPV antigen (Chardonnet *et al* 1986). It remains to be shown whether HPV interacts directly with LC resulting in their cytolysis. Alternatively cytokines secreted by HPV-infected KC, such as TNF-α, may influence LC migration. It is also possible that infection with different types of HPV may cause differences in cytokine modulation. It should be noted that 6/8 warts analysed in this study were facial lesions which are likely to be sun exposed. Irradiation with UVB light decreases LC numbers in human skin, and also modulates normal cytokine and adhesion molecule expression. It is unknown what effect UVB exposure over long periods of time might have on HPV infection. However it has been noted that RAR who are clinically immunosuppressed have increased incidence of HPV infections which develop into squamous cell carcinomas in sun exposed sites (reviewed by Benton *et al* 1992).

3.5.2 Adhesion assay

3.5.2.1 Adhesion of CD3+ and CD18+ T cells to cutaneous warts

Recirculating memory T cells form an important part of the skin immune system and are necessary to promote effective immunity against epidermal pathogens such as HPV. There is evidence that their recruitment is not random (Picker, 1994). In skin, CLA and its ligand, E-selectin, have been implicated in the movement of memory T cells into cutaneous sites (Picker *et al* 1991; Berg *et al* 1991) and there is also evidence that ICAM-1 plays a role in the epidermotropism of LFA-1+ cells (Viac *et al* 1992b). In chronic inflammatory skin diseases such as psoriasis, the specific recruitment may be overridden by a non-specific influx of cells into the tissue. The significantly augmented adhesion of CD3+ and CD18+ cells to psoriatic dermis and epidermis seen in this study and by others (Barker *et al* 1992) reflects the increased trafficking into the involved site. Up regulation of vascular ICAM-1 and E-selectin expression was found in cutaneous warts together with an increased dermal T cell infiltrate compared with normal skin.

The present study investigated whether there is increased adhesion of CD3+ and CD18+ cells to warts compared with normal skin. The results show that there was not increased adhesion to either wart dermis or epidermis compared with adhesion to normal skin. This could reflect the lack of increased trafficking *in vivo* into the HPV lesion. However, the lack of an increase in adhesion to the dermis or epidermis of warts contrasts with the immunohistological findings. The reduced adherence of activated T cells to both the dermis and epidermis of cutaneous warts may reflect reduced functional adhesion to ligands other than ICAM-1 or E-selectin. HPV infection may modulate the expression of cytokines in the local environment and thus may play a role in the observed decrease in functional adhesion. Indeed, many viruses express cytokines (so called virokines) and cytokine receptors which have the potential to modulate normal immune responses (Spriggs, 1994).

3.5.2.2 Role of ICAM-1 in adhesion to cutaneous warts

The contradiction between the immunohistological results and functional adhesion assay may indicate that the adhesion in this assay is not ICAM-1 dependent. Adhesion of $CD3^+$ T cells to tissue sections was partly blocked by monoclonal antibodies to LFA-1 and ICAM-1 but the reduction was never greater than 50%. This suggest that ICAM-1 and LFA-1 adhesion is not solely responsible for the binding of $CD3^+$ T cells to the skin sections in this assay and may reflect the situation *in vivo*. Interestingly there was a lack of ICAM-1 expression on epidermal KC of active psoriatic lesions and wart lesions despite expression in adjacent vascular endothelium which is in apparent contrast with the functional studies. The increased adhesion of $CD3^+$ T cells to psoriasis epidermis, in conjunction with increased numbers of endogenous T cells in the epidermis may indicate; 1) ICAM-1 is not solely responsible for the epidermotropism of T cells in this disorder, and 2) ICAM-1 is not wholly responsible for the adhesion seen in this assay. Indeed Barker *et al* (1992) also found that blocking antibodies to ICAM-1 and LFA-1 did not entirely prevent adhesion to psoriasis sections.

Other adhesion molecules in skin, such as E-selectin, are induced on vascular endothelial cells and play a role in the active movement of cells from the dermal blood vessels into the dermis. LFA-1 also binds to ICAM-2, which could be responsible in part for movement of LFA-1 positive cells from the blood vessels into the dermis. Indeed ICAM-2 was expressed on the vascular endothelial cells in warts in this study. The $\alpha 4$ integrin, VLA-4, and its ligand VCAM-1, although not investigated in this study, is often absent from vascular endothelium in cutaneous inflammation (Picker, 1994). This may suggest that this pathway is not the main pathway for lymphocyte homing to the skin. Other molecules such as the $\alpha 3\beta 1$ integrin on lymphocytes and epiligrin in the basement membrane have been implicated in movement of T cells towards the epidermis (Wayner *et al* 1993). CD44 is also a potential candidate for mediating adhesion since it has a broad

distribution and is the main cell surface receptor for hyaluronic acid, a component of the extra cellular matrix and may be important in lymphocyte homing to inflamed skin (Jalkanen *et al* 1990). Stimulation via CD44 also increases adhesion via the LFA-1 -ICAM-1 pathway (Bruynzeel *et al* 1993). Cytokine expression at a local level is also likely to play a role in movement of leucocytes into the epidermis. There is evidence for the involvement of IL-1, IL-6, IL-8 and MCP-1 in leucocyte trafficking (Bacon *et al* 1990; Westwick *et al* 1989; Gillitzer *et al* 1993).

3.6 Conclusion

Non-regressing cutaneous warts have a $CD3^+$ memory T cell infiltrate in the dermis but not the epidermis. This implies a defect in the epidermal trafficking of T cells, some of which may be HPV-specific. Although epidermal ICAM-1 expression was not observed, ICAM-1 expression on KC may not be necessary for this movement, since it is sometimes not observed in conditions where there are many intraepidermal T cells. The adhesion of activated T cells to wart sections was not increased to levels above that seen for normal skin whereas adhesion to psoriasis skin was increased. It is possible that inflammatory skin, such as psoriasis, may exhibit increased expression of adhesive ligands other than ICAM-1, which are not present in cutaneous warts. Increased vascular expression of ICAM-1 and E-selectin may be responsible for the transendothelial migration, although the involvement of molecules such as VCAM-1or ICAM-2 is also a possibility. It is not known whether the decrease in LC numbers seen in cutaneous warts is a cause or a consequence of prolonged HPV persistence. If the reduction in numbers of LC is a contribution to HPV persistence, then it may be speculated that the 2/8 warts with high LC densities were about to regress. Regressing mucocutaneous lesions have shown increased KC ICAM-1, increased epidermal T cell infiltrate with $CD4^+$ T cells predominating and increased HLA-DR expression. All of these changes may be required for resolution to occur, but it remains unknown what factors initiate the regression of warts.

Chapter 4

Cytokine mRNA expression in cutaneous warts

4.1 Introduction

Although the mechanisms underlying wart regression remain unclear, the resolution of HPV-3 (or 10) associated plane warts is accompanied by a dense mononuclear infiltrate into the epidermis (Tagami *et al* 1980), resembling features of a DTH response (Berman *et al* 1977). Clinical observations on the regression of cutaneous warts differ depending on morphological classification of the wart. For example, during regression of plane warts, erythema and swelling of the warts occurs before they rapidly disappear (Berman *et al* 1977). In contrast to this dramatic resolution of plane warts, similar features have not been described for the regression of common warts or deep plantar warts which frequently become painful and show signs of haemorrhaging before complete resolution. This could suggest a unique regression mechanism for plane warts. However, Coleman *et al* (1994) observed a significant increase in numbers of infiltrating lymphocytes and macrophages in the epithelium of regressing genital warts consistent with a DTH like response, indicating a similar mechanism of cell-mediated regression to plane warts. Increased numbers of infiltrating MHC class II^+cells were also seen in regressing Shope rabbit papillomas, compared with progressing warts (Okabayashi *et al* 1991), which supports the hypothesis that common mechanisms bring about regression in warts infected with different papillomavirus types.

The cytokine network is central to the generation of an inflammatory response in the skin and the recruitment of effector cells to the epidermis, thereby controlling skin immunity. A defect in this cytokine network may explain the inability of mononuclear cells to enter the epidermis of warts and thus clear the virus. Therefore

the aim of this study was to determine if HPV can modulate cytokine mRNA expression in long-term HPV infection, and therefore explain the persistence of warts. Expression of cytokines with a role in induction and regulation of inflammatory processes were examined in cutaneous warts.

4.1.1 Transforming growth factor-β

Transforming growth factor-β (TGF-β) is a multifunctional molecule which can regulate KC growth and the deposition and degradation of extracellular matrix. This cytokine can have a pro-inflammatory role acting as a chemoattractant for monocytes, neutrophils and T cells or can have a suppressive role in inflammation, partly by inducing IL-1 receptor antagonist (IL-1ra) and inhibiting IL-1 receptor (IL-1R) expression (reviewed by Wahl 1994). TGF-β has been found to reduce HPV-16 or 18 early gene transcription in immortalised cell lines *in vitro* (Braun *et al* 1992; Woodworth *et al* 1990). There is also evidence for a decrease in TGF-β mRNA in HPV-induced condylomata (Arany *et al* 1993), and an increase in EV lesions (Majewski *et al* 1991). However, no change in TGF-β mRNA expression in HPV-16/18 immortalised cell lines was observed *in vitro* (Braun *et al* 1992).

4.1.2 Tumour necrosis factor-α

The pro-inflammatory cytokine TNF-α induces the secretion of IL-1(Kutsch *et al* 1993), IL-8, IL-6 and GM-CSF by KC. TNF-α also stimulates the expression of MHC class II, IL-2 receptor and adhesion molecules ICAM-1 and E-selectin on endothelial cells. As discussed in chapter 3, the expression of endothelial adhesion molecules enables granulocytes, monocytes and lymphocytes to bind to the vessel walls and subsequently migrate into the tissues. TNF-α is implicated in the migration of LC from the epidermis (Cumberbatch *et al* 1992). Although expressed at low levels in normal skin (Oxholm *et al* 1991) TNF-α mRNA is raised in EV lesions (Majewski *et al* 1991), and HPV-16 containing tumourigenic cell lines (Malejczyk *et al* 1992). This suggests that TNF-α is up-regulated in HPV infection.

However, Woodworth *et al* (1993) found a reduced secretion of TNF-α in HPV-16 or 18 cell lines immortalised *in vitro*.

4.1.3 Granulocyte macrophage-colony stimulating factor

GM-CSF is produced by KC, T cells, macrophages, mast cells and endothelial cells (Schrader, 1994). It enhances the viability and antigen presentation function of dendritic cells (Witmer-Pack *et al* 1987), and stimulates the migration of leucocytes into the skin with promotion of their survival and phagocytic activity. Although GM-CSF has been shown to be secreted in reduced amounts in HPV-16 or 18 immortalised KC cell lines (Woodworth *et al* 1993), its expression has not been studied in cutaneous warts.

4.1.4 Interleukin-1

IL-1 is a pro-inflammatory cytokine secreted by many cell types with a pivotal role in epidermal inflammation. It induces adhesion molecules on endothelial cells and stimulates the secretion of secondary cytokines such as IL-6 (Partridge *et al* 1991), IL-8 (Larsen *et al* 1989) and GM-CSF (Kupper *et al* 1988) from many cells, including fibroblasts and KC. KC produce active IL-1α and the inactive IL-1β precursor. Unstimulated KC store large amounts of pre-formed IL-1α (Sauder *et al* 1982) which is released upon damage to the epidermis. Since IL-1α is a potent initiator of inflammation in the epidermis several regulatory mechanisms occur. The biological actions of KC-derived IL-1 relies upon the binding of IL-1α to type 1 IL-1 (signalling) receptors, which are found in low numbers on KC, thus regulating the autocrine actions of IL-1α, and epidermal inflammation. Autocrine action of IL-1α on KC is antagonised by IL-1ra which is thought to remain intracellular in KC since it lacks the signal peptide of the secreted form seen in monocytes (Bigler *et al* 1992). KC IL-1ra binds preferentially to type 1 IL-1 receptors, and IL-1α binds preferentially to type 2 IL-1 receptors found in large numbers on the KC cell surface

or shed into the extracellular environment (Groves *et al* 1995).

4.1.5 Interleukin-8

IL-8 is a chemoattractant for neutrophils and T cells, produced by KC, PBMC, dermal fibroblasts and endothelial cells (Matsushima *et al* 1989). This chemokine is a secondary cytokine and its expression is induced by primary cytokines such as IL-1 and TNF-α. Human KC produce IL-8, which plays a role in the movement of T cells and neutrophils towards the epidermis (Barker *et al* 1991). Woodworth *et al* (1993) observed decreased secretion of IL-8 in HPV-16 or 18 immortalised KC cell lines. The expression of IL-8 has not been studied in cutaneous warts.

4.1.6 Interleukin-10

IL-10 or cytokine synthesis inhibitory factor is regarded as an inhibitory regulator of the immune system. It regulates the inflammatory immune response by inhibiting the synthesis of IL-1α, TNF-α, GM-CSF, and colony stimulating factor (CSF) (de Waal Malefyt *et al* 1991) and γ-IFN production by T cells (Fiorentino *et al* 1991; Enk, 1994). The latter may be partially mediated by the down regulation of IL-12 p40 by IL-10 (D'Andrea *et al* 1993). IL-10 steers the immune response towards a T_H2 type response by inhibiting T_H1 function and augmenting T_H2 cell growth (Gajewski *et al* 1988). Increased expression of IL-10 therefore could play a role in the persistence of HPV disease, by promoting an inappropriate T_H2 response as observed in infection with some retroviral and parasitic infections, (reviewed by Sher *et al* 1992). In the skin IL-10 is induced in contact sensitivity responses in mice (Enk *et al* 1992) and may have a role in limiting the inflammatory response induced by this treatment. Exposure of murine KC *in vitro* to UVB induces IL-10 expression and thus suppresses DTH responses in mice (Rivas *et al* 1992). However, the production of IL-10 in human KC *in vitro* remains controversial since some investigators have identified IL-10 protein and mRNA in UVB exposed human KC (Grewe *et al* 1995; Enk *et al* 1995), while others could not demonstrate the

expression of the IL-10 gene (chapter 5 and Ried *et al* 1994; Teunissen *et al* 1995).

4.1.7 Interleukin-12

IL-12 is a hetero-dimer of p35 and p40 molecules; p35 is constitutively expressed in many cell types, whereas the p40 is inducibly regulated. The IL-12 hetero-dimer produced by monocytes, macrophages, neutrophils, KC, B-cells and LC is potent at inducing the production of γ-IFN from NK and T cells. The combination of γ-IFN and IL-12 shifts immune responses towards a cellular or T_H1 response (Trincheri, 1993). KC express p35 constitutively and IL-12 p40 can be induced upon contact sensitisation (Muller *et al* 1994). IL-12 can also counteract the immune suppression observed following UVB irradiation (Schmitt *et al* 1995).

4.1.8 γ-Interferon

Memory $CD4^+$ T cells can be divided into T_H1 or T_H2 subsets in murine and human systems depending on their cytokine secretion profile (Mosmann *et al* 1986; Kapsenberg *et al* 1991). T_H1 subsets produce IL-2, γ-IFN and IL-12 thus promoting macrophage functions and inhibiting B cell help. T_H2 secrete cytokines not produced by T_H1 cells including IL-4 and IL-10, thus promoting B cell help and inhibiting macrophage function. Therefore humoral and cell mediated immunity are often mutually exclusive. Gamma interferon is also produced by NK cells and has been detected in B cells (Dayton *et al* 1992), macrophages (Fultz *et al* 1993) and fibroblasts (Rady *et al* 1995). Gamma IFN induces MHC class II on many cell types (Barker *et al* 1989) and can induce ICAM-1 on KC and endothelial cells.

4.1.9 Interleukin-4

The T_H2 T cell subset produce IL-3, 4, 5, 6 and 10. IL-4 production is typical of a T_H2 type $CD4^+$ T cell which stimulates immunoglobulin synthesis and drives a T_H2 response. Detection of IL-4 in warts will indicate the presence of activated T cell populations of the T_H2 subset.

4.1.10 Amphiregulin

Amphiregulin (AR) is a member of the EGF family which can have either growth promoting or inhibitory effects on epithelial cells (Plowman *et al* 1990). As for EGF and transforming growth factor-α , AR is synthesised as a transmembrane precursor, with the secreted protein being released after proteolytic cleavage. AR is the main autocrine growth factor for KC (Piepkorn *et al* 1994), acts via the EGF receptor and is induced by insulin and insulin-like growth factor-1 *in vitro* (Vardy *et al* 1995). AR mRNA is upregulated in psoriasis (Cook *et al* 1992), and immunostaining of AR *in vivo* correlates with epidermal proliferative activity (Piepkorn *et al* 1995). AR has been localised to the nucleus and is proposed to have DNA binding activity (Modrell *et al* 1992), thus indicating a direct role for AR in controlling gene expression. Significantly, Woodworth *et al* (1995), showed that IL-1α induced AR which mediated growth promotion in a cervical carcinoma derived HPV-16 containing cell line.

The expression of cytokines in HPV infection *in vitro* and *in vivo* is variable and may depend on HPV type, site of infection, or degree of malignancy. Cytokine expression has not been previously studied in cutaneous warts and was therefore investigated in this study. Preliminary non-quantitative RT-PCR experiments were performed to determine whether TGF-β, TNF-α, GM-CSF, IL-1α, IL-1β, IL-10 , IL-8 and IL-12 p40 mRNA were expressed in cutaneous warts. Since there appeared to be reproducible differences in the expression of some cytokines, semi-quantitative RT-PCR mRNA expression of IL-1α, intracellular IL-ra (icIL-1ra), secretory IL-1 ra (secIL-1ra), IL-10 , IL-8, IL-12 p40 and AR was then investigated in non-regressing cutaneous warts and compared with expression in normal skin. The house-keeping gene glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) was used throughout to normalise the expression of each cytokine, as described previously (Kondo *et al* 1993),

4.2 Methods

4.2.1 Patients

Non-regressing viral warts from 24 patients between the ages of 18 and 68 years were removed by curettage as part of clinical management of these patients. Half of each wart was formalin-fixed for routine histological examination, and the remainder was snap frozen in liquid nitrogen and stored at -70^{o}C. Eleven of the warts were common hand warts, one a common wart in the nostril, 2 mosaic, 4 deep plantar, 1 plantar mosaic, 1 deep palmar wart, 1 plane wart, and 3 common warts from renal allograft recipients. The duration of these warts varied from 2 months to 30 years. Samples of normal skin used in this study were taken from 4 breast reductions and 7 foreskins (taken from donors of up to 11 years old which were removed for non-medical reasons) and were snap frozen until processed. Ethical permission for this study was granted following review by a local ethics committee, and was carried out in accordance with standards laid down in the 1964 declaration of Helsinki.

4.2.2 RNA extraction and reverse transcription.

Frozen tissue specimens were ground into a fine powder in a mortar and pestle with liquid nitrogen, before lysis in 4M guanidinium isothiocyanate. Total RNA was extracted (Chomczynski *et al* 1987), quantitated spectrophotometrically and 1 μg total RNA was used as a template to generate cDNA. RNA was denatured at 65^{o}C for 5 min, before adding the reverse transcription (RT) cocktail consisting of: 5 mM $MgCl_2$; 1 X RT buffer (10 mM tris HCl, 50 mM KCl, 0.1% triton X-100); 1 mM each dinucleotide triphosphate (dNTP); 2.5 units RNAse inhibitor; 15 units avian myeloblastosis virus reverse transcriptase; 0.5 μg oligo $dT_{(15)}$ in a reaction volume of 20 μl. After incubation for 1 hr at 42^{o}C the reaction was terminated by heating to 95^{o}C for 5 min. All reagents were from the Promega RT system (Promega Corp, Madison, WI, USA).

4.2.3 Primers

Table 4.1 PCR primers pairs

Cytokine	Primer sequence	Product size (bp)
IL-1α	5' =5' ATGGCCAAAGTTCCAGACATGTTT3' 3' = 5' GGTTTTCCAGTATCTGAAAGTCAG3'	816
IL-1β	5' =5'AAACAGATGAAGTGCTCCTTCCAG3' 3' =5'TGGAGAACACCACTTGTTGCTCCA	388
TNF-α	5' =5'ATGAGCACTGAAAGCATGATCCGG3' 3' = 5'GCAATGATCCCAAAGTAGACCTGCCC3'	695
TGF-β	5' = 5'CGCCTTAGCGCCCACTGCTCCTGTGT3' 3' = 5'GGGGCGGGACCTCAGCTGCACTTG3'	573
IL-4	5' = 5'ATGGGTCTCACCTCCCAACTGCT3' 3' = 5'CGAACACTTTGAATATTTCTCTCTCAT3'	456
γ–IFN	5' = 5'GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC3' 3' = 5'CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG3'	427
GM-CSF	5' = 5'ATGTGGCTGCAGAGCCTGCTGC3' 3' =5'CTGGCTCCCAGCAGTCAAAGGG3'	424
IL-8	5' = 5'ATGACTTCCAAGCTGGCCGTGGCT3', 3' = 5' TCTCAGCCCTCTTCAAAAACTTCTC3'	289
IL-10	5' =5'AAGCTGAGAACCAAGACCCAGACATCAAGGCG3' 3' =5'AGCTATCCCAGAGCCCCAGATCCGATTTTGG3'	328
IL-12p40	5'=5'CTACTTCTCCCTGACATTCT3' 3'=5'TGGTCTATTCCGTTGTGTC3'	269
icIL-1 ra	5' = 5'CAGAAGACCTCCTGTCCTATGAGG3' 3' =5'TTGTCAGGCATATTGGTGAGGCTGAC3'	470
secIL-1 ra	5' = 5'TTCGTCAGGCATATTGGTGAGGCTGAC3' 3' = 5'ATGGAAATCTGCAGAGGCCTC3'	492
G3PDH	5' = 5'CATGTGGGCCATGAGGTCCACCAC 3' 3' = 5'TGAAGGTCGGAGTCAACGGATTTGGT3	983
AR	5' = TCCTCGGGAGCCGACTATGAC 3' 3' = GGACTTTTCCCCACACCG 3'	329

Primer pairs spanned at least one intron to distinguish cDNA product from genomic DNA. IL-1β (Krishnaswamy *et al* 1993), TNF-α (Krishnaswamy *et al* 1993), TGF-β (Van Zoelen *et al* 1993), IL-8 (Krishnaswamy *et al* 1993), IL-12p40 (Zhang *et al* 1994) and AR (Ebert *et al* 1994) were synthesised by Oswell DNA (Southampton, UK), icIL-1ra, (Chan *et al* 1992) secIL-1ra (Chan *et al* 1992) and GM-CSF (Lagoo-Deenadayalan *et al* 1993) were synthesised by Dalton Chemical labs (Ontario, Canada), and IL-1α, G3PDH, IL-10, IL-4, γ-IFN were from Clontech (Palo Alto, CA, USA).

4.2.4 Controls

Positive controls for cytokine expression were RNA from PBMC stimulated with PHA (1μg / ml) and PMA (50 ng / ml) (Sigma) for 24 hrs. For IL-10 and IL-12 p40, 4 hr stimulated PBMC and a bladder carcinoma cell line (SD) were used respectively. MCF-7 cell line stimulated with 100 ng / ml PMA for 24 hrs provided the positive control for AR (Plowman *et al* 1990). Negative controls were cDNA synthesis reactions of RNA from positive controls minus reverse transcriptase enzyme in order to rule out the possibility of amplifying genomic DNA. PCR reactions with H_2O instead of cDNA were also used to check for contamination of PCR reagents.

4.2.5 PCR amplifications

PCR was performed using 1.5 μl RT reaction in a PCR cocktail containing 2.7 mM $MgCl_2$, 0.2 mM d NTPs (Promega), 5 units *Thermus aquaticus* (Taq) thermally stable DNA polymerase (Promega) 1 X PCR buffer (10 mM Tris HCl, 50 mM KCl, 0.1% Triton X -100) and 0.16 μM of each primer in a reaction volume of 23 μl. All PCR reactions were hot started (D'aquila *et al* 1991) and run on a Techne PHC3 dri-block cycler (Techne, Cambridge, UK) at 94°C for 45 sec, 60°C for 2 min, 72°C for 3 min.

4.2.6 Non-quantitative PCR using Southern blots

4.2.6.1 Blotting.

Preliminary non-quantitative PCR was performed as above for 30 cycles to detect mRNA for IL-1α, IL-1β, IL-8, TNF-α, TGF-β, IL-10, IL-12 p40, γ-IFN, IL-4 and G3PDH. PCR products were resolved by agarose gel (1.5%) electrophoresis which were denatured and neutralised as described previously (Sambrook *et al* 1989) before blotting onto positively charged nylon membrane (Zeta probe GT, Biorad, Hemel Hempstead, UK) by capillary action in 10 X sodium saline citrate (SSC) buffer overnight. Blots were rinsed in 2 x SSC before fixing for 1 minute face down

on a UV transilluminator, air dried and stored at room temperature.

4.2.6.2 Generation of probes

Digoxigenin-labelled riboprobes were generated by subcloning cytokine cDNA fragments into the Bluescript SKII plasmid using standard methods (Sambrook *et al* 1989). Sense or anti-sense RNA run-off transcripts were generated from this template in the presence of either T3 or T7 RNA polymerase (Promega). Plasmids containing cDNAs for the cytokines investigated were provided by : Dr. A Singh, Genentech CA, USA. (TGF-β and TNF-α), Dr. P. Lomedico, Hoffman -La Roche, NJ. USA. (IL-1α), Dr. S. Wolf, Genetics Institute Inc. MA, USA. (IL-12 p40), Dr. G. Wong, Genetics Institute Inc. MA, USA (GM-CSF), Dr. J. Oppenheim, National Cancer Institute, MD, USA. (IL-8) and IL-10 cDNA was cloned and sequenced from a PCR product using a TA cloning kit (Invitrogen Inc., The Netherlands). Bluescript plasmids containing cytokine cDNA inserts were linearised by restriction digest, using enzymes which did not leave 3' overhangs. Linearised plasmids were subjected to agarose gel electrophoresis to confirm the completion of the reaction. The linear plasmid was then phenol / chloroform extracted, precipitated to remove enzyme and any other contaminating proteins and resuspended in H_2O. One μg of linearised plasmid DNA was then used as a template to generate a digoxigenin labelled riboprobe in 1 x RNA labelling mix (1 mM ATP, 1 mM GTP, 1 mM CTP, 6.5 mM UTP, 3.5 mM DIG-UTP) (Boehringer Mannheim, Lewes, UK), 1 X transcription buffer (Promega) (20 mM Tris pH 7.5, 3 mM $MgCl_2$, 1 mM spermidine, 5 mM NaCl), 20 units RNAse inhibitor (Promega), 40 units T3 or T7 RNA polymerase (Promega), 10 mM dithiothreitol (DTT) (Promega) in a reaction volume of 20 μl. Labelling reactions were incubated at 37°C for at least 1 hr before stopping the reaction with 2.5 μl 200 mM EDTA, and precipitating at -70°C overnight with 2.5 μl 4M LiCl, 100 μl 100% ethanol, before resuspending in 20 μl H_2O plus RNAse inhibitor.

4.2.6.3 Confirmation of probe labelling

Under the conditions specified, 1 µg DNA linear template was estimated to generate 10 µg of labelled riboprobe (Boehringer Mannheim Ltd, 1993). The efficiency of the digoxigenin labelling was confirmed by: 1) subjecting a sample of probe to agarose gel electrophoresis, blotting the gel and detecting the digoxigenin as described below, 2) dilutions of the probe from 10 ng / µl down to 0.1 pg / µl were made and 1 µl dots were spotted on to membrane followed by digoxigenin detection (section 3.2.6.4). The limit of detection was 1 pg of probe.

4.2.6.4 Digoxigenin detection

Detection of digoxigenin was performed by incubating the membrane in 1% blocking reagent (Boehringer), for 30 min, followed by 1/5000 anti-digoxigenin alkaline phosphatase F(ab) fragments (Boehringer) diluted in blocking reagent for 30 min. Membranes were washed in Buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5) 2 x 15 min, and then developed without shaking in 10 ml colour solution (10 ml Buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), 45 µl nitro-blue tetrazolium (Sigma), (100 mg/ml in 70% dimethylformamide), 35 µl 5-bromo-4-chloro-3-indolylphosphate (Sigma), (50 mg / ml in dimethylformamide) in the dark for up to 2 hrs. Membranes were finally rinsed in 10 mM Tris 1 mM EDTA and air dried in the dark.

4.2.6.5 Hybridisation

Membranes were pre-hybridised at 55°C for at least 30 min in high SDS buffer (7% SDS, 50 mM $NaPO_4$ pH 7.0, 50% formamide, 2% blocking reagent (Boehringer), 5 x SSC, 0.1% sarkosyl) using a Hybri-tube (Gibco-BRL), before hybridising in 100 ng / ml riboprobe in high SDS buffer overnight at 55°C. Stringency washes were as follows:- 2 x 5 min at room temperature in 2 X SSC, 0.1 % SDS, followed by 2 x 15 min at 55°C in 0.2 X SSC, 0.1 % SDS. Membranes were rinsed in Buffer 1 before developing as described above. One riboprobe was used for all hybridisations for

each cytokine, and stored at -20^{o}C between use. Each probe was confirmed as being specific for each cytokine before use.

4.2.7 Semi-quantitative PCR

4.2.7.1 Labelling primers

For semi-quantitative PCR, each primer was ^{32}P-end labelled in a reaction mix consisting of 5 μM primer, 1 X polynucleotide kinase (PNK) buffer (70 mM Tris HCl, 10 mM $MgCl_2$, 5 mM DTT) 0.5 units T4 PNK (New England Biolabs, Beverly, MA, USA) and 0.05 μl 7000 Ci / mmol γ [^{32}P] -dATP (ICN Biochemicals, Thame, UK) at 37^{o}C for 1 hour before denaturing at 95 ^{o}C for 5 min.

4.2.7.2 Radiolabelled PCR

PCR was performed as described in section 3.2.5 except 1.5 μl radiolabelled primer (5' and 3' mixed) were added last to 20 μl PCR cocktail containing 1.5 μl of RT reaction mix at 85^{o}C (hot start). Reactions were overlaid with 20 μl of mineral oil to prevent evaporation. The optimum number of cycles were derived for each primer pair by plotting product yield against cycle number following autoradiography. The cycle number for further amplifications was chosen from a point within the linear exponential portion of the plot.

4.2.7.3 SDS-PAGE and autoradiography

Radioactive ^{32} P-labelled PCR products were subjected to 12 % SDS-PAGE at 150 volts for 1.5 hr. The gels were exposed to Kodak X Omat AR autoradiography film (Sigma). Autoradiographs were visualised on the Seescan system image analyser (Seescan, Cambridge, UK) and integrated OD units were obtained using gel analysis software v1.0 1D (Seescan). The housekeeping gene G3PDH was used to control for differences in abundance of cDNA in RT reactions (Tso *et al* 1985). The ratio of integrated OD units for each cytokine : G3PDH, were used to compare relative

cytokine mRNA expression in warts compared with normal skin samples. All warts and normal skins and controls were amplified within the same run, subjected to SDS-PAGE and autoradiographed at the same time. All PCR reactions for each tissue sample used cDNA from identical RT reactions. All PCR reactions were performed at least twice.

4.2.8 Statistical Analysis

Ratios of cytokine : G3PDH integrated OD units derived from image analysis of autoradiographs were subjected to statistical analysis. The Mann-Whitney U test for unpaired samples was used on Systat version 5.2.1 for the Macintosh. P values of less than 0.05 were considered significant.

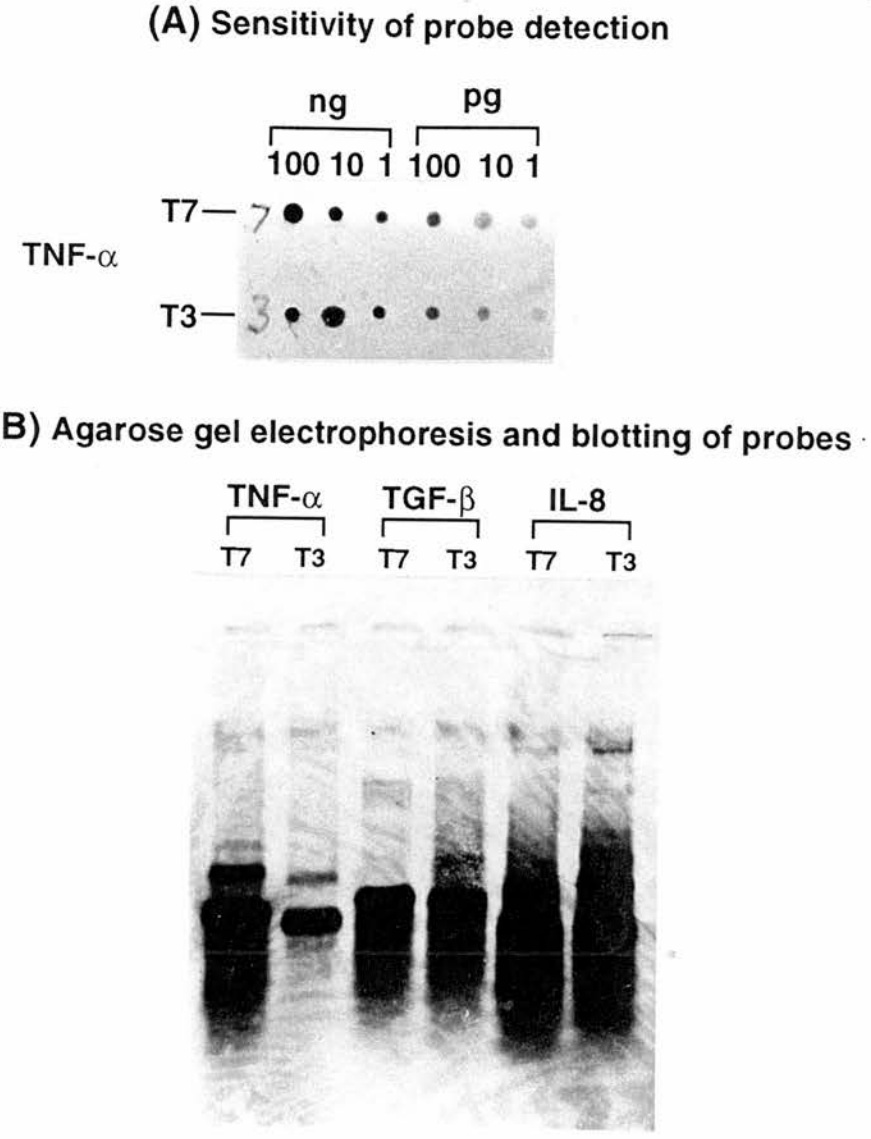
4.3 Results

4.3.1 Preliminary Non Quantitative PCR

4.3.1.1 Confirmation of probe digoxigenin labelling and specificity

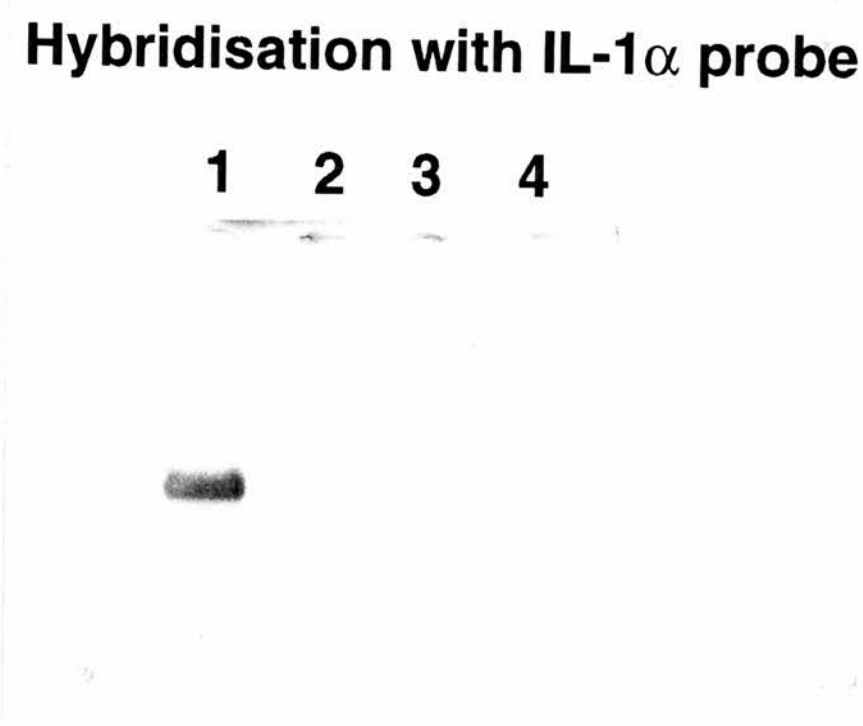
Before hybridisation of membranes, confirmation of digoxigenin labelling was performed and the specificity of each riboprobe was confirmed. One-ten pg of riboprobe could be detected for all the probes used in this study (Figure 4.1 A) and were visualised on blotted agarose gels (Figure 4.1 B). To confirm the specificity of each probe, hybridisations were performed with blots of a range of PCR product targets. Figure 4.2 indicates that the riboprobe generated from the IL-1α cDNA template was specific for IL-1α PCR product. Non-specific hybridisation was not detected to PCR products from G3PDH, GM-CSF or IL-1β primers. The specificity of all probes were confirmed before use.

Figure 4.1 Digoxigenin labelling of probes



To confirm that riboprobes had been labelled dilutions of T7 and T3 RNA polymerase generated TNF-α riboprobes were dotted onto nylon membrane (A). In addition riboprobes (TNF-α, TGF-β and IL-8) were run on 1% agarose gels, and blotted onto nylon membrane (B). Digoxigenin was detected as described in section 4.2.6.4.

Figure 4.2 Confirmation of specificity of riboprobes

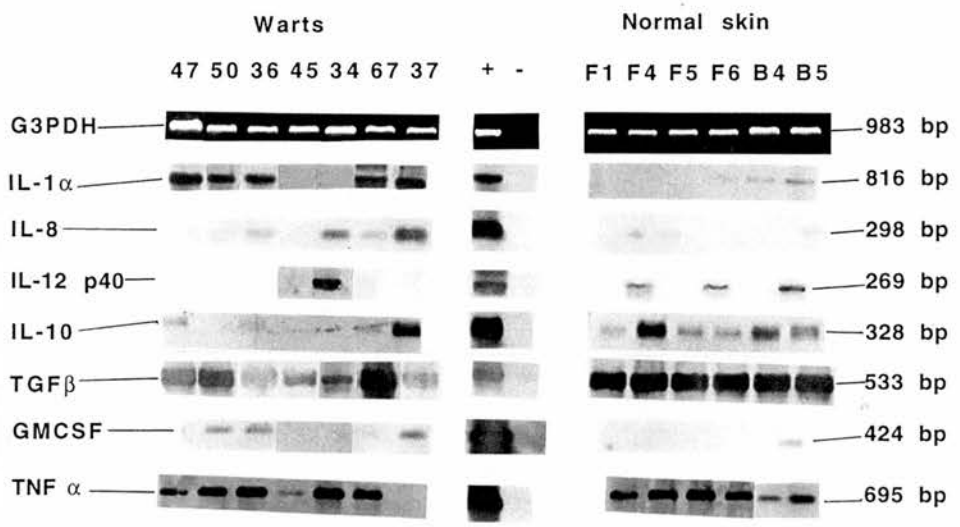


Southern blot of PCR products described in section 4.2.6.1 for IL-1 α (lane 1), IL-1β (lane 2), G3PDH (lane 3) and GM-CSF (lane 4) hybridised with the probe generated from the IL-1α cDNA template described in section 4.2.6.2

4.3.1.2 Non-quantitative cytokine expression in warts and normal skin

In preliminary experiments, expression of TNF -α, TGF-β, GM-CSF, IL-10, IL-8, IL-12 p40, γ-IFN, IL-4, IL-1β and IL-1α mRNA was investigated in cutaneous warts (n=18) and normal skin (n=10) using RT- PCR. Electrophoresis of PCR products on agarose gels and Southern blotting using specific digoxigenin-labelled riboprobes was carried out as described in section 4.2.6. In the majority of cases cytokine PCR products were only detectable after Southern blotting. An indication of the abundance of a particular cytokine mRNA was obtained depending on whether the product could be detected by ethidium bromide staining or only by more sensitive Southern blotting.

Figure 4.3 Cytokine expression in warts and normal skin



Southern blots of PCR products are shown for 6 warts and 6 normal skin samples, with positive and negative controls (+ and -).

The housekeeping gene G3PDH was expressed in all tissue samples included in this study, indicating that RNA was intact and that RT-PCR had been performed successfully. The percentages of warts and normal skin samples which expressed each cytokine were calculated and are shown in Table 4.2.

Preliminary studies indicated the following trends: 1) cutaneous warts may have higher levels of IL-1α mRNA expression compared with normal skin, 2) IL-10 mRNA was constitutively expressed in normal skin as judged by Southern blotting, whilst fewer numbers of warts expressed this cytokine (although 2/18 warts expressed IL-10 at higher levels than normal skin), 3) Fewer numbers of warts expressed IL-12 mRNA than normal skin. No marked differences were apparent in the numbers of warts expressing GM-CSF, TGF-β, TNF-α, or IL-1β mRNA compared with normal skin.

Table 4.2 Percentages of warts and normal skin samples expressing mRNA for each cytokine

	Warts		Normal Skin	
Cytokine	Ethidium bromide	Southern blotting	Ethidium bromide	Southern blotting
TNF-α	67%	89%	90%	100%
TGF-β	89%	100%	100%	100%
IL-1α	56%	83%	0%	90%
IL-1β	56%	ND	50%	ND
IL-8	0%	72%	20%	50%
IL-10	11%	72%	0%	100%
IL-12 p40	33%	33%	50%	70%
GM-CSF	11%	61%	0%	70%
γ-IFN	0 %	ND	0%	ND
IL-4	0 %	ND	0%	ND

The percentage of warts and normal skin samples containing detectable mRNA of each cytokine following agarose gel electrophoresis and Southern blotting. (warts n = 18, normal skin n = 10)

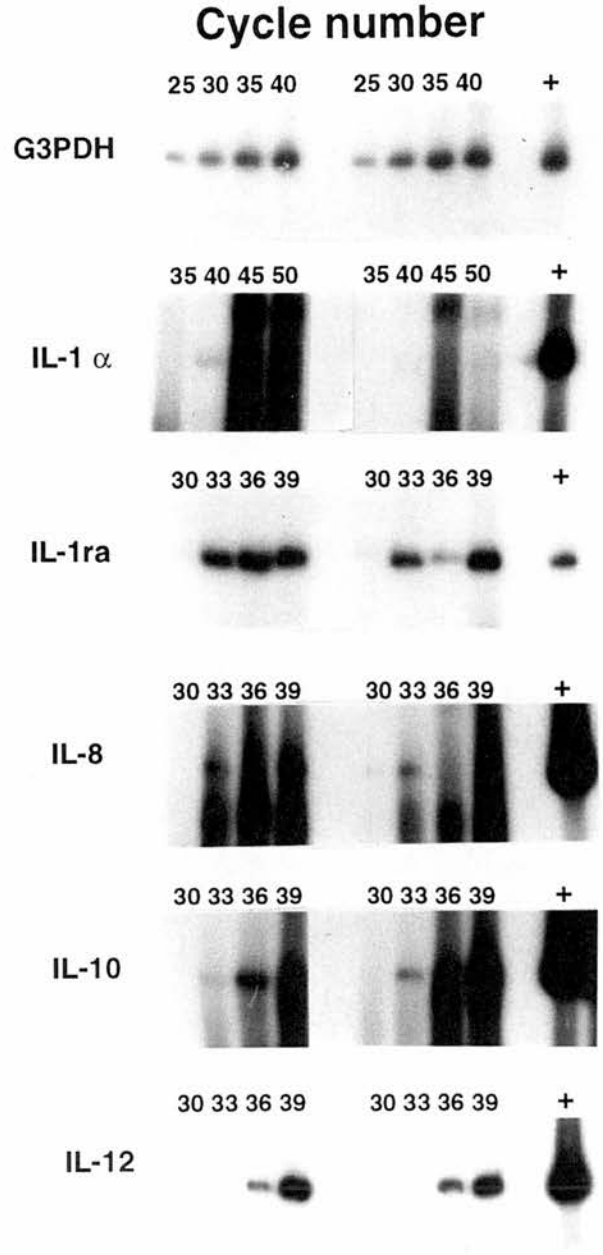
ND = not done.

4.3.2 Semi-quantitative PCR

4.3.2.1 Optimum cycle number determination

In order to analyse cytokine mRNA expression semi-quantitatively, the optimum cycle number for each PCR product was determined. A pool of cDNAs taken from wart and normal skin RT reactions expressing high and low amounts of each cytokine mRNA were used as target sequences.

Figure 4.4 Determination of optimum cycle numbers

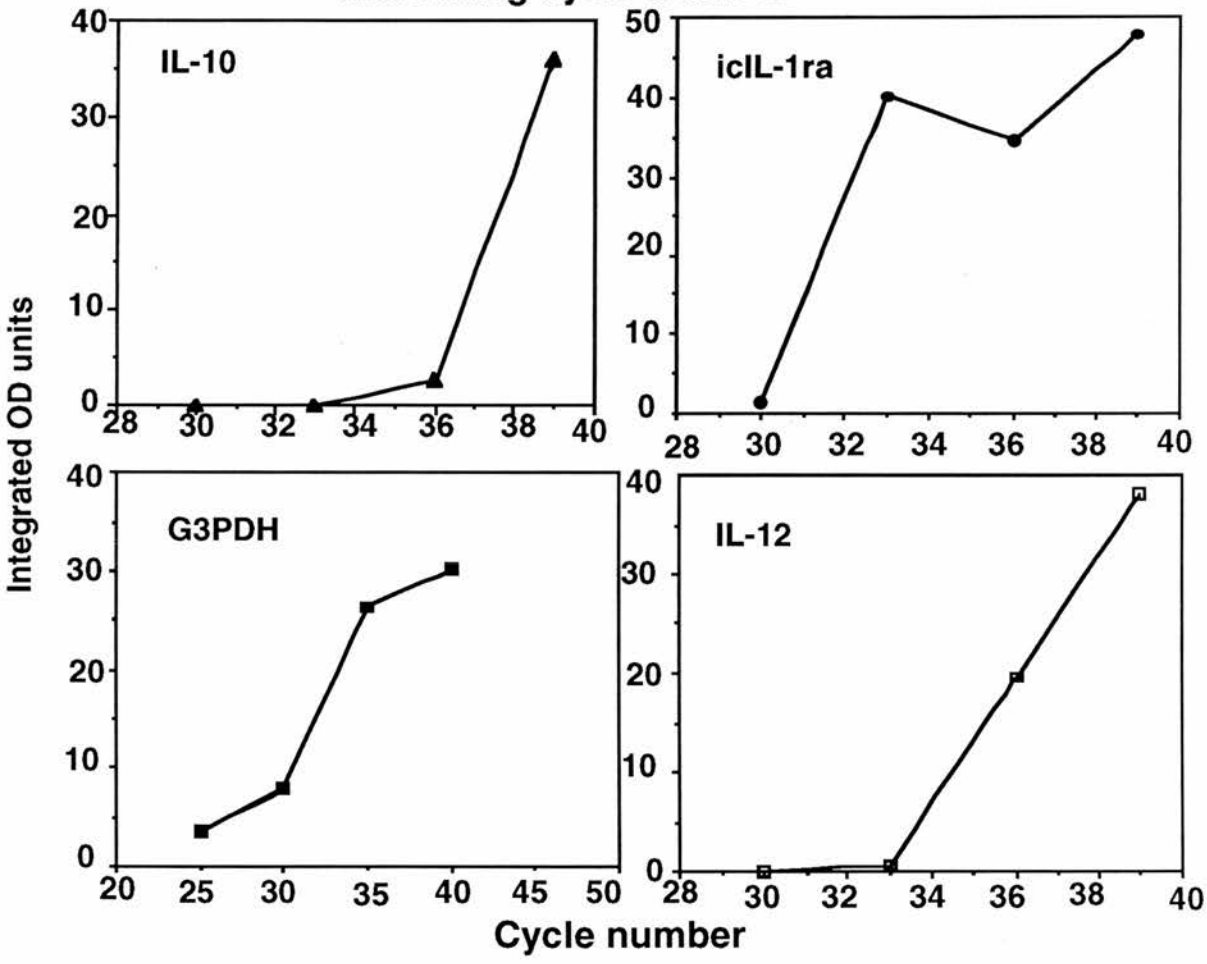


Eight identical PCR reactions were established for each cytokine as described in section 4.3.2.1. The PCR was performed, removing 2 reaction tubes at a determined cycle number. Products were subjected to SDS-PAGE and autoradiographs of these gels are shown.

Figure 4.4 shows that a product for IL-1α could only be detected after 40 PCR cycles. A specific PCR product was not obtained beyond this cycle number, therefore 40 cycles were routinely used to detect IL-1α mRNA. PCR cycle

numbers of 33-39 generated a smear when detecting IL-8 mRNA. Thirty nine cycles were used to detect IL-8 mRNA in further experiments which generated a specific product (Figure 4.6). SecIL-1ra mRNA could not be detected in any wart or normal skin sample (data not shown) using 40 cycles. Forty cycles was the maximum cycle number used in this study since above this number the reaction is likely to plateau due to exhaustion of Taq and PCR reagents. Integrated OD units of PCR products from these cycle experiments were derived where possible and the linearity of PCR product accumulation versus cycle number was determined (Figure 4.5).

Figure 4.5 Linearity of PCR product accumulation with increasing cycle number



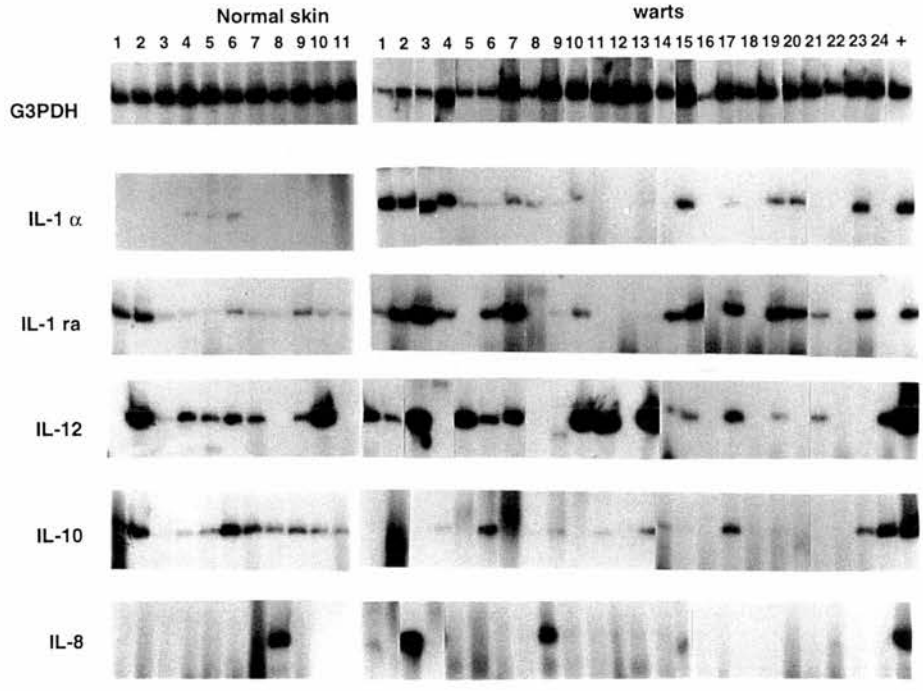
Densitometry of PCR product shown in Figure 4.4 was performed where possible and accumulation of PCR product versus cycle number is shown.

Optimum cycle numbers for detection of each cytokine mRNA determined from Figures 4.4 and 4.5 were 40 cycles for IL-1α, 36 cycles for IL-10, 39 cycles for IL-8, 36 cycles for IL-12 p40, 31 cycles for icIL-1ra, 40 cycles for secIL-1ra and 35 cycles for G3PDH.

4.3.2.2 Semi-quantitative expression of cytokines in warts and normal skin

The expression of IL-1α, icIL-1ra, IL-8, IL-10 and IL-12 p40 mRNAs was analysed in non-regressing cutaneous warts and compared with normal skin samples. Autoradiographs of PCR products for these cytokines at the optimum cycle numbers are shown in Figure 4.6.

Figure 4.6 Cytokine expression in warts and normal skin



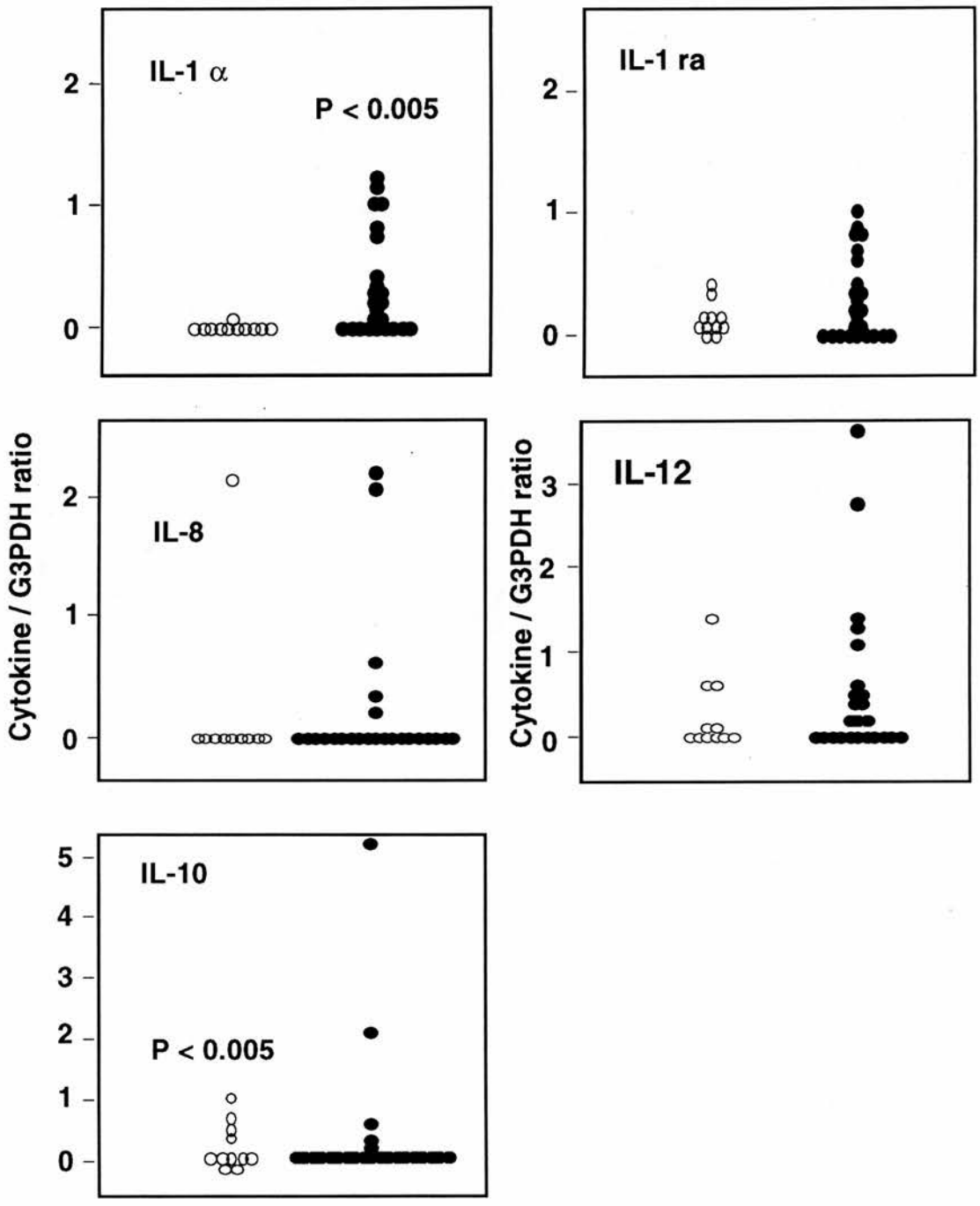
Autoradiographs of ^{32}P labelled cytokine PCR products for 11 normal skin and 24 warts were subjected to SDS-PAGE and the results are shown above. Warts 1-3, 8 and 24 were plantar , 6, 7, 9, 10, 12- 14, 17-19 and 23 were common hand warts, while 15 and 16 were mosaic, 20-22 were common warts taken from renal allograft recipients, 11 was a plane wart, 4 was a common wart from the nostril and 5 was a deep palmar wart.

IL-1 α mRNA in the normal skin samples and many warts was either absent or present at low levels, whereas 33% warts expressed this cytokine at high levels. The majority of warts which expressed IL-1α mRNA also expressed icIL-1ra mRNA whereas most normal skin samples expressed this mRNA. The secreted form of IL-1ra was not expressed in any of the normal skin samples or warts, but a PCR product was obtained when mRNA was used from PBMC stimulated for 4 hr with PHA and PMA (data not shown). Expression of mRNA for secreted IL-1ra has previously been shown in monocytes and fibroblasts but is not found in KC. IL-12 p40 mRNA was expressed at varying levels in warts and normal skin but no marked difference in expression was apparent between the two skin types. IL-10 mRNA was present at low levels in all normal skins but was not detected in many warts, thus confirming the findings of the preliminary experiments. IL-8 mRNA was detected in one normal skin sample, while 5 warts expressed mRNA for this cytokine. However, 3 of these warts had a low abundance of IL-8 PCR product. Densitometry was performed on the autoradiographs and ratios of integrated OD units for cytokine: G3PDH are shown in Figure 4.7. Many warts have increased IL-1α, icIL-1ra and IL-12 p40 mRNAs compared with normal skin (Figure 4.7). The expression of IL-1α mRNA was significantly greater in warts than normal skin ($p = 0.003$), while the expression of IL-10 mRNA was significantly greater in normal skin than in cutaneous warts ($p = 0.002$).

4.3.2.3 IL-1α mRNA expression in common and plantar warts

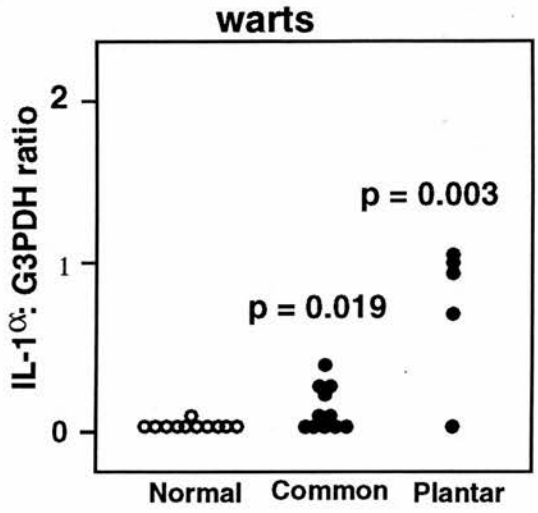
Differences in IL-1α mRNA levels could occur depending on anatomical site, morphology and HPV type. Although molecular HPV typing of warts was not available for this study, the anatomical site and clinical diagnosis was known. When common and plantar warts were compared, only the expression of IL-1α mRNA was significantly different between these wart types (shown in Figure 4.8).

Figure 4.7 Expression of cytokine mRNAs in warts and normal skin



Integrated OD units were obtained by image analysis of autoradiographs. Relative expression of each cytokine is shown as a ratio of cytokine : G3PDH. Normal skin = O, warts = ●, IL-1α mRNA expression was significantly greater in warts than normal skin. IL-10 mRNA was significantly greater in normal skin (median = 0.200) compared with warts (median = 0.000).

Figure 4.8 IL-1α mRNA expression in common and plantar warts



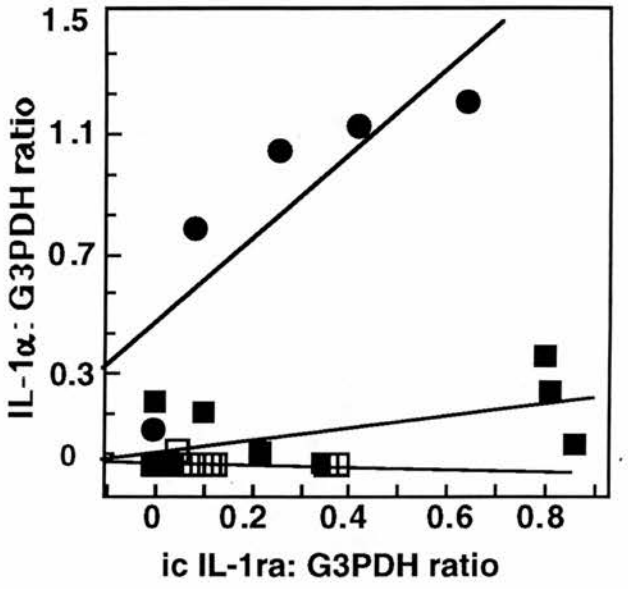
Ratios of IL-1α : G3PDH mRNAs expression are compared for normal skin (n = 11) O , common warts (n = 11) ● , and plantar warts (n= 5) ● , and are shown above.

Significantly greater IL-1α mRNA expression was found in common hand warts than in normal skin (p = 0.019). Plantar warts also had significantly greater IL-1α expression than common warts (p = 0.003). IL-1α was the only cytokine which had greater expression levels in warts than normal skin, and had differential expression depending on wart type. Of the three warts taken from RAR, one expressed IL-1α, icIL-1ra and IL-10 mRNAs, one expressed icIL-1ra and IL-12 mRNAs while the third did not express any of the cytokines studied. The one plane wart expressed IL-12 and IL-10 mRNAs.

4.3.2.4 Correlation between IL-1α and icIL-1ra mRNA expression

The autoradiographs in Figure 4.6 indicate that warts which expressed IL-1α mRNA also expressed icIL-1ra mRNA. The correlation between the expression of these mRNAs is shown in Figure 4.9.

Figure 4.9 Correlation between IL-1α and icIL-1ra mRNA expression



IL-1α / : G3PDH ratios were correlated with icIL-1ra : G3PDH ratios Normal skin = □ , Common warts = ■ , Plantar warts = ●. Pearson correlation r values are 0.546 for common warts, - 0.180 for normal skin and 0.831 for plantar warts which was the only significant correlation (p = 0.05).

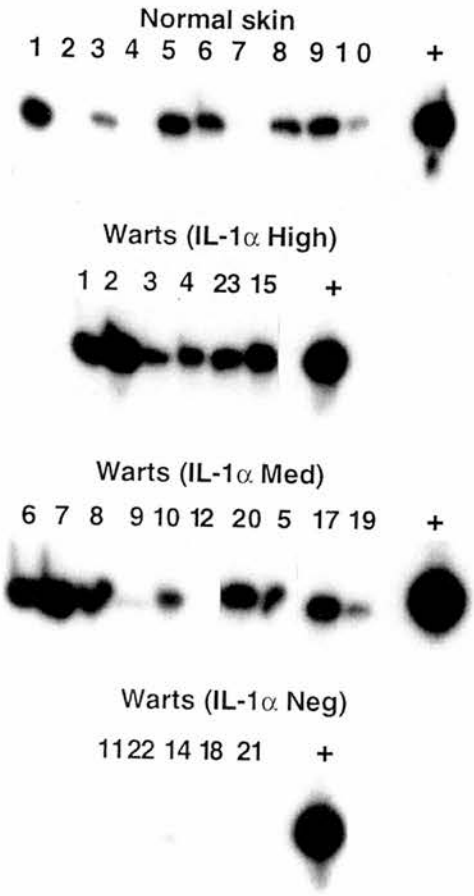
There was no correlation between the expression of mRNA for IL-1α and icIL-1ra in samples from normal skin. Indeed, icIL-1ra mRNA was present in normal skin samples but IL-1α mRNA was absent or at low levels. There was a trend toward correlation between IL-1α and icIL-1ra mRNA levels in common warts, but the expression of these 2 mRNAs was not significantly correlated. In samples of plantar warts the correlation between icIL-1ra and IL-1α mRNA expression was significant (Pearson correlation r = 0.846, significant at p = 0.05).

4.3.2.5 Amphiregulin mRNA expression in cutaneous warts

Since IL-1α mRNA was significantly up-regulated in cutaneous warts compared with normal skin, the expression of AR mRNA in cutaneous warts was examined.

Warts were divided into groups of high, medium and negative for IL-1α mRNA expression based on levels found in semi-quantitative RT-PCR shown in Figure 4.7. Warts in the negative category were also negative by Southern blot. Semi-quantitative RT-PCR analysis of AR expression was performed, the autoradiographs are shown in Figure 4.10. Warts which expressed either medium or high levels of IL-1α also expressed AR

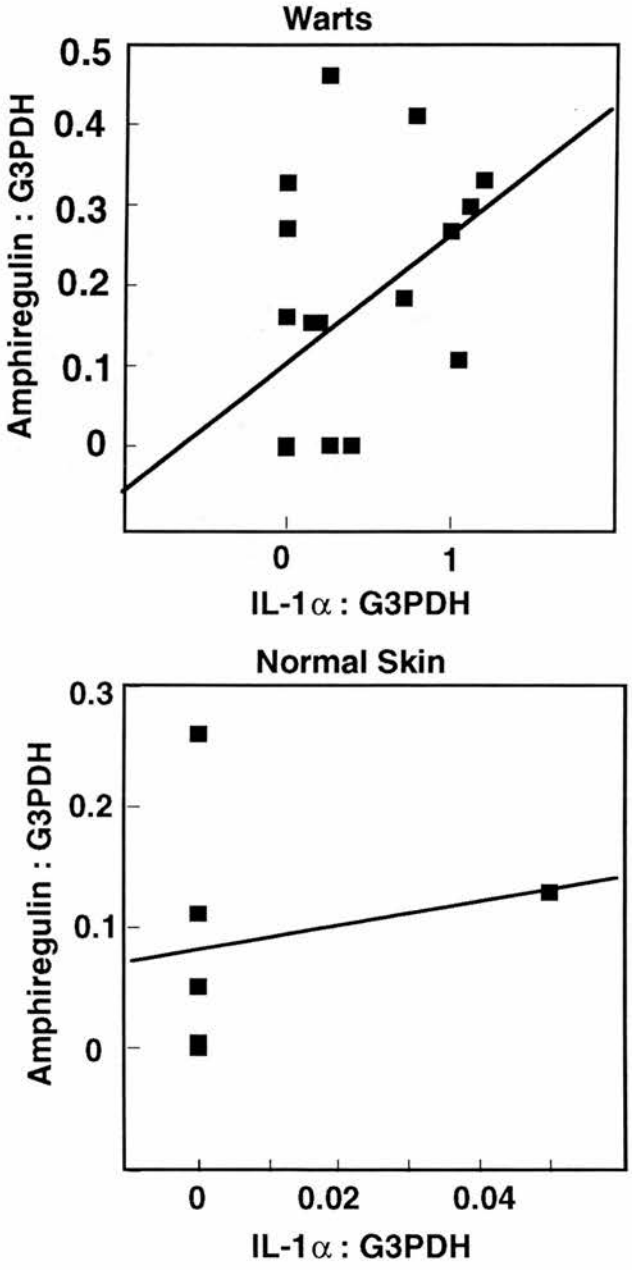
Figure 4.10 Amphiregulin expression in cutaneous warts



Autoradiographs of ^{32}P-labelled amphiregulin PCR products separated by SDS-PAGE. IL-1α high = 6 warts with the highest level of IL-1α mRNA expression as shown in Figure 4.7. IL-1α Med = all other warts in which IL-1α was detected. IL-1α Neg = warts in which no IL-1α was detected (either with ^{32}P labelled primers or by Southern blotting).

mRNA, while warts negative for IL-1α mRNA expression did not express AR mRNA. The expression of AR mRNA was positively correlated with IL-1α mRNA expression in warts (Pearson correlation $r = 0.590$, $p < 0.05$), shown in Figure 4.11. No such correlation was observed in normal skin.

Figure 4.11 Correlation of amphiregulin and IL-1α mRNA expression



IL-1α / : G3PDH ratios were correlated with AR : G3PDH ratios in normal skin and cutaneous warts. A positive correlation ($p < 0.05$) was found between AR and IL-1α expression in cutaneous warts.

4.4 Discussion

The primary aim of this study was to investigate whether cytokine expression is modulated in chronic cutaneous HPV infection. Preliminary non-quantitative experiments found that more warts expressed IL-1α and IL-8 than normal skin, while fewer warts expressed IL-10 and IL-12. No marked differences were found between the numbers of warts and normal skin samples expressing TNF-α, TGF-β, IL-1β or GM-CSF. No IL-4 or γ-IFN mRNAs were found at levels detectable by RT-PCR and visualised by agarose gel electrophoresis in either warts or normal skin. Semi-quantitative analysis found significantly higher IL-1α mRNA expression in warts than normal skin, whereas IL-10 mRNA was found at lower levels in warts than normal skin.

4.4.1 HPV infection and TGF-β

TGF-β mRNA was found abundant normal skin, as shown previously (Arany *et al* 1993; Majewski *et al* 1991). There was no change between the percentages of normal skin samples and warts expressing TGF-β mRNA by non quantitative RT-PCR analysis. TGF-β mRNA has been reported to be increased in HPV-5 or 8 associated EV lesions (Majewski *et al* 1991) and decreased in HPV-6 or 11 associated condylomas (Arany *et al* 1993). This discrepancy in TGF-β expression could be due to different effects of the HPV types causing the infection or differing host responses at cutaneous and mucosal epithelium. However, the induction of TGF-β mRNA seen in EV lesions was greater in the samples showing signs of malignancy suggesting an association between TGF-β mRNA induction and progression towards carcinoma (Majewski *et al* 1991). Although expression of TGF-β mRNA may indicate that bioactive TGF-β is potentially available in cutaneous warts, since the gene is regulated at both transcriptional and post translational level (reviewed by Wahl, (1994) the functional protein may not be present. Furthermore, since nascent secreted TGF-β is biologically inactive, due to

its interaction with a latency associated protein and activation of the latent TGF-β complex must occur before TGF-β is available to bind to cellular receptors, (reviewed by Wahl 1994).

HPV immortalised KC *in vitro* have been shown in some studies to be resistant to growth inhibition by TGF-β (Pietenpol *et al* 1990). However, not all HPV containing cell lines are resistant to TGF-β (Braun *et al* 1992; Woodworth *et al* 1990). HPV containing cell lines derived from carcinomas or high passage number cultures were resistant to TGF-β mediated growth inhibition (Braun *et al* 1992). Taken together these findings indicate that increased TGF-β mRNA expression and resistance to its growth inhibitory effects may play a role in progression towards carcinogenesis rather than in the persistence of HPV infection. TGF-β could mediate regression of pre-neoplastic HPV lesions in the early stages of infection, and the subsequent loss of responsiveness to TGF-β may aid the progression to carcinoma.

4.4.2 HPV infection and TNF-α

Similar numbers of normal skin samples and wart specimens expressed TNF-α mRNA in this study. Although all normal skin samples and the majority of warts expressed TNF-α mRNA, in agreement with previous data (Viac *et al* 1995), 2 warts were found not to express TNF-α mRNA. It is postulated that TNF-α has a role in the migration of LC from the epidermis (Cumberbatch *et al* 1992). Most cutaneous warts show a reduced density of LC in the epidermis compared with normal skin, but this reduction is not universal (Chapter 3). It has not been determined if the warts lacking TNF-α mRNA expression in this study are those without a reduction of LC in the epidermis. Many cell types secrete TNF-α including KC, T cells and macrophages. It has been suggested that the TNF-α produced by infiltrating mononuclear cells has a role in wart regression, rather than the TNF-α production by

KC (Hagari *et al* 1995). The phenotype and location of TNF-α expressing cells was not addressed in this study.

TNF-α mRNA is elevated in EV lesions (Majewski *et al* 1991) and HPV-16 containing KC cell lines (Malejczyk *et al* 1992), but reduced secretion was found in other HPV-16 containing cell lines (Woodworth *et al* 1993). TNF-α inhibits growth of normal human skin KC lines (Pillai *et al* 1989), KC derived from normal cervix (Woodworth *et al* 1995) and HPV-16 transformed KC *in vitro* (Malejczyk *et al* 1992; Villa *et al* 1992). Nevertheless, resistance to growth inhibitory effects of TNF-α has also been observed in HPV-16, 18 and 33 containing KC (Delvenne *et al* 1995; Villa *et al* 1992; Malejczyk *et al* 1994). In other cases TNF-α promoted the growth of HPV-16/18 immortalised KC (Woodworth *et al* 1995). In common with the loss of TGF-β growth inhibition outlined above, escape from TNF-α growth inhibition is associated with increased tumourigenicity of the cell lines (Malejczyk *et al* 1994).

A decrease in the TNF-α receptor has been observed in HPV-16 tumourigenic cell lines and also increased secretion of soluble TNF-α receptor type 1 (Malejczyk *et al* 1994), which suggests antagonism of TNF-α in these cell lines. Although TNF-α, similar to TGF-β, may be involved in the progression to carcinoma of HPV-16 containing KC lines, the data presented in this study would suggest that this cytokine is not modulated in chronic infection with cutaneous HPV types. However, TNF-α may still have a role in the regression of cutaneous warts, as indicated in CRPV warts (Hagari *et al* 1995), which may be due in part to its role in reducing HPV transcription (Kyo *et al* 1994)

4.4.3 HPV infection and GM-CSF

GM-CSF mRNA was expressed in 2/18 warts and 0/10 normal skin samples, when PCR products were visualised on agarose gels. However, no marked difference in the numbers of wart samples or normal skin samples expressing GM-CSF mRNA was seen when Southern blotting was performed. GM-CSF is not constitutively produced in normal skin, but upon stimulation, monocytes, endothelial cells, fibroblasts and KC are able to express this cytokine. The low level of GM-CSF transcripts detected here may represent untranslated cytokine in both normal skin and warts. The only other report to date concerning GM-CSF and HPV infection is the study by Woodworth *et al* (1993) which detected reduced secretion of GM-CSF in HPV-16 and 18 immortalised KC cell lines, suggesting a down-regulation of GM-CSF by HPV. The data from the present study do not support these findings.

4.4.4 HPV infection and IL-1

Preliminary studies showed a greater percentage of warts expressing IL-1α mRNA than the percentage of normal skin samples. No difference in the percentage of warts or normal skin samples expressing IL-1β mRNA was observed. Semi-quantitative RT-PCR analysis confirmed these preliminary findings and indicated that IL-1α mRNA expression was significantly ($p = 0.003$) greater in warts compared with normal skin. In addition, plantar warts expressed significantly more IL-1α mRNA than common hand warts.

Although RT-PCR is a very sensitive technique, enabling large numbers of samples to be analysed, it does not yield information regarding phenotype of the cells expressing cytokine mRNA in cutaneous warts. KC comprise the majority of cells within the curetted specimens of warts and are the main producers of IL-1α within the epidermis. However, a small amount of dermis is included; therefore infiltrating mononuclear cells, and dermal fibroblasts which also express IL-1α could be a source of IL-1α mRNA.

Protein expression or biological activity of IL-1α needs to be analysed for a definitive interpretation of these PCR results. Bioactive IL-1α is stored pre-formed in epidermal KC and is released upon damage or trauma which could include infection with papillomavirus. Many of the warts in this study have been present for a number of years, therefore continuous up-regulation of bioactive IL-1α seems unlikely since these lesions rarely shown signs of inflammation. Indeed, cutaneous warts typically show few signs of being inflammatory lesions. Although ICAM-1 and E-selectin are up-regulated on vascular endothelium in cutaneous warts in conjunction with a mononuclear infiltrate into the dermis (chapter 3).

IL-1α is a potent initiator of inflammation, and a network regulating the actions of IL-1 exists within the epidermis. KC and fibroblasts produce IL-1ra (Chan *et al* 1992) which inhibits the action of IL-1 *in vitro* by binding to type 1 receptors (Bigler *et al* 1992). The mRNA for the secreted form of this molecule found in monocytes and fibroblasts was not detected in this study, but the mRNA for the intracellular form found in KC was expressed in both normal skin and warts. Many IL-1α expressing warts also showed icIL-1ra mRNA expression, therefore negative regulation of IL-1α is possible. Since IL-1 induces IL-8 expression in PBMC, KC, dermal fibroblasts and endothelial cells (Matsushima *et al* 1989), the lack of IL-8 mRNA in many warts which express IL-1α mRNA, may indicate regulation of bioactive IL-1α. The ratio of IL-1ra to IL-1α is an important indicator of bioactive IL-1α. A ratio of IL-1ra : IL-1α is approximately 100 to 1 is found in uninfected normal skin (Hammerberg *et al* 1992).

Cutaneous warts which expressed IL-1α mRNA also tended to express AR mRNA, and a significant correlation was observed between the two. No correlation was found between the expression of AR mRNA and IL-1α mRNA in normal skin. IL-1α promotes the growth of HPV-16 or 18-transformed KC *in vitro*, mediated by AR

(Woodworth *et al* 1995), but IL-1α remains inhibitory to the growth of normal KC. Although a causal relationship cannot be assumed from mRNA expression in cutaneous warts, one speculation is that IL-1α may induce the observed AR mRNA expression in cutaneous warts and thus promote the growth of HPV-infected KC. Woodworth *et al* (1995) showed that AR stimulated growth of HPV infected KC through interaction with the EGF receptor. Interestingly the papillomavirus E5 protein associates with the EGF receptor (Pim *et al* 1992) preventing its degradation and increasing the sensitivity of the cell to EGF and therefore increasing cell proliferation. This data indicates that KC in benign cutaneous HPV infections, may have increased sensitivity to AR. In some cases, such as condylomata, this effect may be augmented by increased EGF receptor expression (Viac *et al* 1990). The E5-EGF receptor interaction may be a first step in the transition to viral mediated transformation, although E6 and E7 later function to maintain this transformed phenotype. A previous study has also shown nuclear localisation of AR and binding to DNA, indicating a possible role in transcriptional regulation (Modrell *et al* 1992).

It is not clear whether the autocrine AR stimulation is associated with integration of the genome of oncogenic types of HPV and immortalisation of the host cells. Autocrine growth stimulation of KC in cutaneous warts could occur which may explain the hyper-proliferative nature of epidermal KC in cutaneous warts. IL-1α is also an inducer of IL-6 which itself induces AR expression in HPV-immortalised KC (Iglesias *et al* 1995). However, in other studies, IL-1α had no effect on the growth of carcinoma cells containing HPV-16 (Malejczyk *et al* 1992).

If IL-1α is not released from KC, it could act in an intracellular autocrine fashion, by binding receptors before they reach the cell surface. Intracellular autocrine action has been described for GM-CSF, IL-6, IL-3 and platelet derived growth factor in immortalised cell lines (Lang *et al* 1990). IL-1α has also been shown to decrease

early viral transcription of HPV-16 immortalised cells (Kyo *et al* 1994) suggesting a role for pro-inflammatory cytokines in the defence against HPV.

4.4.5 HPV infection and IL-8

IL-8 mRNA was detected in more warts than normal skin samples by both Southern blot and ^{32}P-labelled detection of RT-PCR products. However, in semi-quantitative analysis, the abundance of IL-8 mRNA was not significantly different in warts than in normal skin when IL-8 : G3PDH ratios were subjected to statistical analysis. This suggests that IL-8 is not modulated by HPV infection in cutaneous warts. The lack of IL-8 expression in many warts may partly explain impaired trafficking of mononuclear cells into the epidermis of warts, and thus viral persistence (chapter 3). Interestingly, HPV-16 immortalised cell lines secrete less IL-8 than normal KC lines (Woodworth *et al* 1993).

4.4.6 HPV infection and IL-10

By both the semi-quantitative RT-PCR and non-quantitative assays, all normal skin samples with the exception of two samples had low level constitutive IL-10 mRNA expression, as observed by others (Teunissen *et al* 1995). The majority of the warts had no IL-10 mRNA but it was induced to high levels in 2 lesions. Normal skin expressed more IL-10 mRNA than warts ($p = 0.002$). It has been suggested that up-regulated IL-10 expression aids viral persistence (Sher *et al* 1992), however these results do not support a similar hypothesis for HPV infection. It could be speculated that IL-10 inhibits expression of cytokines in normal skin, thus its absence in the majority of cutaneous warts might lead to increased expression of IL-1α, IL-8, IL-12 cytokines and IL-1ra. However, there is no evidence from other studies to suggest that this is the case.

No data on localisation of IL-10 mRNA expression are presented in this study. It is

possible that KC are not the main source of IL-10 in human skin. Indeed infiltrating monocytes have been proposed as the main epidermal source of IL-10 (Kang *et al* 1994). In human KC IL-10 mRNA could not be detected in some *in vitro* studies (Ried *et al* 1994; Teunissen *et al* 1995). However, the present study found IL-10 mRNA without the detection of protein in human KC exposed to high doses of UVB irradiation (200 J / m^2) (Chapter 5). The role of KC derived IL-10 in human skin is still unclear since other studies have found low levels of IL-10 mRNA in human KC lines, and varying levels of protein (Grewe *et al* 1995; Enk *et al* 1995). Nickoloff *et al* (1994) detected KC derived IL-10 protein in human skin *in situ* after tape stripping and in poison ivy dermatitis.

4.3.7 HPV infection and IL-12

Many cell types are known to constitutively express IL-12 p35. However, the expression of IL-12 p40 is restricted to certain cell types, including KC. These data presented in this study, indicate that many warts and normal skin samples have increased IL-12 p40 mRNA expression. A trend towards higher relative expression of IL-12 p40 mRNA was observed in cutaneous warts compared with normal skin, but these differences were not significant ($p > 0.05$).

Monocytes and KC are the primary secretors of IL-12 in the skin and there is evidence for this cytokine having a role in primary immune responses in the skin. IL-12 is known to stimulate NK cells which are important in immunosurveillance at cutaneous sites. Individuals with HPV-16 associated neoplasms had decreased NK cell activity, but whether this is reflected in a decreased IL-12 secretion by the PBMC of these patients is unclear (Malejczyk *et al* 1994). The presence of the IL-12 p40 mRNA in cutaneous warts suggests that the IL-12 protein may be present in the wart lesion. IL-12 is able to induce the expression of cutaneous lymphocyte antigen (Leung *et al* 1995) which increases skin-specific homing. Cells infiltrating the underlying dermis of cutaneous warts may be activated T_H1 cells, but will be ineffective if these cells are still unable to reach the epidermis.

4.3.8 HPV infection and γ-IFN

No γ-IFN mRNA could be detected in either normal skin or warts in this study, indicating that long-term infection with cutaneous HPV types does not modulate expression of this cytokine. The absence of this cytokine may play a role in wart persistence. Gamma-IFN inhibits growth of normal KC and HPV-transformed cells *in vitro* (Delvenne *et al* 1995), and has been shown to reduce HPV gene transcription in some studies (Woodworth *et al* 1992) but not in others (Kyo *et al* 1994). The present study provides no evidence that HPV modulates γ-IFN expression during long-term cutaneous infection.

4.3.9 HPV infection and IL-4

No IL-4 mRNA could be detected samples from warts or normal skin, indicating a lack of activated T_H2 cells secreting IL-4 in cutaneous warts. These data do not support the theory that chronic cutaneous HPV infection may develop or persist as a result of an inappropriate T_H2 response which is unable to resolve infection.

4.3.10 The role of UVB in HPV infection

The common warts examined in this study were obtained from hands or face and are thus exposed to UVB radiation, which may influence the outcome of the infection at these sites. UVB exposure leads to systemic immunosuppression in mice and decreased DTH reactions. Pre-exposure of human KC *in vitro* to UVB prevents induction of MHC class II and ICAM-1 by γ-IFN (Khan *et al* 1993; Krutmann *et al* 1990). Individuals who are clinically immunosuppressed presumably have decreased cutaneous immunosurveillance and are particularly susceptible to infection with HPV (reviewed by Benton *et al* 1992). UVB exposure also predisposes to the development of cutaneous warts in RAR (Boyle *et al* 1984) and many of these warts develop into squamous cell carcinomas (Benton *et al* 1992). Some of the HPV types in these lesions were found to be HPV 5, 8 or similar types

associated with EV, a rare genetically determined disease characterised by extensive flat wart-like lesions on sun exposed areas. Up to 33% of EV patients develop multiple SCC, and 90 % of these lesions contain HPV 5 or 8 DNA. UVB exposure may act as a co-factor in the development of cancer in HPV types with an oncogenic potential. However, there are also cases where warts resulting from infection with HPV-1 were transformed into squamous cell carcinoma in immunosuppressed patients (Noel *et al* 1994), suggesting that the low risk HPV types may have the potential to promote progression to squamous cell carcinoma when other co-factors are present.

4.5 Conclusion

This is the first study investigating cytokine mRNA expression in cutaneous warts. The up-regulation of IL-1α mRNA occurs in non-regressing cutaneous warts compared with normal skin. There are two potential roles for IL-1α in HPV infected skin. Firstly, IL-1α may begin the chain of events leading to inflammation in the epidermis, but is blocked at some point down stream of transcription. Hence, the lack of infiltrating effector cells into the epidermis. Secondly, it may play a role in the persistence of the virus by maintaining growth of the infected KC, by induction of KC growth factors such as AR. Therefore IL-1α which is often considered to assist immune responses may actually aid progression of infection with HPV.

The limitations of this study are that the cytokine localisation and protein expression remain un determined. The advantages of this study are that cytokine expression was measured *in vivo* , thus removing any *in vitro* artefacts which may occur when measuring cytokine expression in cell lines. Previous studies found a reduced secretion of cytokines in HPV infected immortalised KC cell lines (Woodworth *et al* 1993). However, normal cervical KC infected with retroviral vectors containing E6 and E7 did not cause modulation of cytokine secretion, suggesting different effects of HPV infection and HPV associated carcinogenesis on cytokine modulation.

Chapter 5
HPV infection of keratinocytes *in vitro*

5.1 Introduction

HPV infection may modulate the epidermal cytokine network and contribute to the impaired trafficking of T cells to the site of infection. Alternatively, lack of cytokine production from intraepithelial lymphocytes (such as γ-IFN or IL-12) may play a part in the persistence of infection. Analysis of cytokine mRNA in cutaneous warts (Chapter 4) demonstrated increased levels of IL-1α mRNA and a correlation with AR mRNA expression. Although these cytokines may not be responsible for the impaired trafficking into the epidermis, they may play a role in the hyperproliferation of HPV-infected KC in cutaneous warts.

KC are the major potential source of cytokines within the skin, and the target hosts for HPV infection, therefore the modulation of KC cytokines may be one way in which HPV modulates the host response to aid its survival and wart persistence. To study the direct effects of HPV on cytokine expression in KC requires an *in vitro* experimental system to model HPV infection. *In vitro*, HPV will only productively infect a stratified epithelium, such as an organotypic raft culture treated with PMA (Dollard *et al* 1992). Such an *in vitro* model provides the ideal situation for analysis of the virus-host interaction. However, valuable information regarding the early stages of KC- HPV interaction may still be gained by studying non-productively infected cells.

The majority of *in vitro* data concerning cytokine production by KC containing HPV comes from cell lines immortalised with oncogenic HPV types. Woodworth *et al* (1993) showed a reduced secretion of GM-CSF, IL-6, IL-8, IL-1β and TNF-α in KC immortalised with HPV-16. Cytokine production was reduced to a greater extent in

cell lines derived from carcinomas but remain unchanged in normal cervical KC infected with retroviral recombinants encoding HPV-16 E6 and E7 oncoproteins (Woodworth *et al* 1993). This suggests that a reduction in cytokine production is due to secondary changes associated with transformation of the cells and progression to carcinomas rather than HPV infection per se. Malejczyk *et al* (1991, and 1992) demonstrated constitutive secretion of IL-6 and TNF-α in tumourigenic HPV-16 containing KC lines. TNF-α has a growth inhibitory effect on normal KC although tumourigenic cell lines are resistant to the growth inhibitory effects of TNF-α. Taken together, these data suggest that changes in cytokine production by KC occur in association with progression to malignancy. Alternatively, long term culture of transformed KC may lead to changes in secretion and response to cytokines. The effect of HPV on host cytokine production in benign infections in non-transformed KC is less clear.

Although productive infection of HPV will not occur in monolayer cultures, KC infected with HPV-1 particles *in vitro* retain HPV DNA up to 4 months (Bossens *et al* 1992; La Porta *et al* 1982). Replication of HPV DNA is detected in monolayers of KC infected with HPV (La Porta *et al* 1982), and some studies have detected E1^E4 transcripts (Chow *et al* 1987). Similar experiments using BPV-1 infection of human monocytes demonstrated BPV DNA up to 28 days after infection (Bonnez *et al* 1991). IL-1α is detected in the supernatant of these cells 3 days after infection. HPV specific RNAs were found in human bronchial epithelial cells infected with HPV-1 particles (Christian *et al* 1987). E1^ E4 mRNAs have also been detected in primary KC cultures from explants of HPV-11 infected normal human foreskin tissue (Smith *et al* 1993). These transcripts could be detected in secondary passaged cells 6 weeks after infection.

Transfection of HPV DNA in KC may mimic early stages of infection. Oncogenic HPV types (HPV-16, 18, 33) immortalise host cells after integration of HPV DNA

into cellular genomes (Durst *et al* 1987; Gilles *et al* 1993). The linearised genomes of HPV-11 and 18 replicate transiently for up to 7 days in squamous cell carcinoma cell lines which do not contain endogenous HPV DNA sequences (Del Vecchio *et al* 1992). "Low risk" HPV genomes, such as HPV-1 or 11, when transfected into KC are retained transiently, generally without integration. When HPV-1 does integrate into the cellular genome *in vitro*, it does not lead to a transformed phenotype (Burnett *et al* 1983). Recircularised HPV-11 DNA, co-transfected into KC cultures with a selectable marker, remains episomal and replicates (Mungal *et al* 1992).

Early stages of HPV infection can be mimicked by infection of KC cultured as monolayers and the cytokine response from infected cells can be studied at various time points after infection to determine the effect of HPV on cytokine expression. Similarly the transfection of HPV-1 DNA provides an opportunity to examine host cell response to viral DNA replication and HPV transcription. If KC are co-transfected with recircularised HPV-1 genome and a plasmid containing a selectable marker, such as neomycin resistance, selection of HPV containing cells is possible. HPV DNA, mRNA and cellular mRNA can then be analysed. In addition assays of cytokine mRNA in KC transiently infected with HPV would provide information regarding the KC response to HPV, and whether this might be altered by UVB irradiation.

5.2 Aim

The aim of this study was to determine whether cytokine mRNA expression in primary human KC *in vitro* is modulated by transient infection with cutaneous HPV types or transfection with HPV DNA.

5.3 Methods

5.3.1 Primary keratinocyte lines

Sterile instruments were used and procedures were performed under sterile conditions. Foreskins excised from boys up to age 11, were obtained from the Western General Hospital, Edinburgh, and placed in Leibowitz medium (Gibco) containing gentamicin (50 μg / ml) and amphotericin-B (50 ng / ml) during transportation. Tissue was washed 5 X in 0.02% EDTA in PBS, retaining the last wash to check for tissue culture sterility. Using a sterile petri dish foreskins were cut along the dorsal axis to form a square. The square of tissue was then trimmed with scissors to remove extraneous fat and blood vessels and cut into 1 cm squares. Approximately 5 tissue squares were placed in 5 ml dispase (4 mg / ml in PBS) (Sigma) and incubated either at room temperature overnight or 4°C for 72 hrs on a rotary mixer. The epidermis was then easily peeled away from the underlying dermis. Epidermal sheets were then placed in 10 ml 0.25% trypsin (Gibco) in PBS / EDTA and vigorously chopped for 3 minutes with scissors to achieve a fine suspension. This suspension was incubated at 37°C for 30 min on a roller mixer. Before the addition of 1 ml filtered foetal calf serum to inhibit the trypsin, the suspension was vigorously pipetted for 2-3 min. To increase the yield in some preparations, the tissue was incubated for a further 15 min at 37°C in 0.4 mg / ml collagenase (Sigma). The suspension was pipetted for a further 2 min before passing rapidly through a stainless steel mesh. The resulting suspension was centrifuged at 1800 rpm for 10 min. The cells were resuspended in 1 ml keratinocyte serum-free medium (KGM) (Clonetics Corp. San Diego, USA) with insulin (5 μg / ml), bovine pituitary extract (0.03 mg / ml), hydrocortisone (0.5 μg / ml), gentamicin (50 μg / ml), amphotericin-B (50 ng / ml), murine EGF (10 ng / ml) and 0.06 mM $CaCl_2$. Viability and cell yield was determined using trypan blue exclusion, the large, angular, terminally differentiated squames were not counted.

Isolated epidermal cells were either stored in liquid nitrogen or dispensed at 1 x 10^6 cells per 25 cm^2 either in T25 flasks (Falcon) with 3 ml KGM or in a 12 well plate (Costar) with 1 ml KGM / well. Tissue culture plastics were coated with 5 μg / cm^2 fibronectin (Scottish National Blood Transfusion Service, Edinburgh, Scotland) in PBS at room temperature for at least 20 min and were stored at -20°C after the fibronectin solution was removed. Fresh medium was added to plates every 2-3 days and cultures were inspected every day for colony formation. KC colonies formed after 2 - 20 days in culture. Once colonies had established, medium was aspirated and replaced with fresh medium as required. Cultures were split 1:3 when 70-80 % confluent. Any cultures which had contaminating fibroblasts were cleansed with PBS / EDTA at 37°C for 2 min since fibroblasts are more sensitive to EDTA treatment than established KC colonies.

5.3.2 Characterisation of cell lines

To confirm that KC cultures consisted of epithelial cells rather than contaminating fibroblasts cells were stained for expression of keratin, vimentin, MHC class I and II and ICAM-1. KC from passage numbers 1-3 were cultures on Thermanox™ plastic coverslips (Nunc) in 6 well dishes at 4 x 10^4 cells per disc in 20-50 μl . After KC became attached 3 ml KGM was added per well and 2-3 days later the discs were air dried and fixed in acetone for 10 min at room temperature before re-drying. Discs were rehydrated into TBS and stained using the immunoperoxidase technique as described in Chapter 3. Primary antibodies were: mouse anti-vimentin 1/200 (Sigma), mouse anti-ICAM-1 1/200 (R and D systems), mouse anti-HLA-DR 1/40 (DAKO), guinea pig anti-keratin 1/40 (Sigma), mouse anti-MHC class I 1/40 (DAKO). Mouse anti-CD3 1/20 (DAKO) was an isotype matched control for ICAM-1, vimentin and MHC class II. Secondary rabbit anti-guinea pig or anti-mouse horseradish peroxidase conjugated antibodies were visualised by developing with diaminobenzidine as described in Chapter 3.

5.3.3 HPV infection of primary keratinocytes

KC cell lines from passage number 1-3 were cultured at 7 x 10^4 cells per well of a 24 well plate (Costar). Cells were grown in the absence of EGF for 48 hrs prior to infection and during the course of the experiment. When 30-50 % confluence, was reached cultures were infected with HPV as described by Bossens *et al* (1992). Briefly, 200 µl HPV (isolated as described in chapter 2, but not UV inactivated) estimated to be approximately 10^8 particles / ml was added per well to cultures in PBS containing 1.8 mM strontium chloride, 0.45 mM $MgCl_2$ for 1 hr at 37°C. Alternatively cultures were mock infected (treated with buffer only). KGM without EGF was replaced onto these cultures and cells were harvested by trypsinisation on days 1 and 3 after infection.

5.3.4 Transfection of KC with plasmid DNA

KC were seeded at 4 x 10^4 per well of a 6 well plate (Costar) in KGM containing all the additives as described above. KC were grown without EGF for 48 hrs before transfection and when 50 -60 % confluent were transfected with a eukaryotic assay vector pCH110 (Pharmacia LTD. Milton Keynes, UK). This plasmid contains a functional *lac* Z gene and its product, β-gal, was assayed to monitor transfection efficiency. Transfections were performed using Lipofectamine™ (Gibco BRL) reagent in KGM containing additives described above (without gentamicin and amphotericin-B) according to the manufacturers' instructions. Briefly, 2 µg pCH110 was mixed with 100 µl KGM (without antibiotics) and incubated for 45 min with 1, 2, 4 or 8 µl Lipofectamine™ diluted in 100µl KGM. This final mixture was placed on KC for 5 hrs before replacing with KGM containing additives without EGF. In addition, an epithelial bladder carcinoma cell line (UMUC-3) was transfected as a positive control since this immortalised cell line can be easily be transfected *in vitro*. β-gal gene activity was assayed 12 hrs later by fixing cultures in 1% glutaraldehyde for 5 min at 4°C followed by 3 washes in PBS. Staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride and 1 mg /

ml X-gal (Gibco stock at 20 mg / ml in dimethyl formamide in PBS) was added to cultures and incubated overnight at 37°C in a humid chamber. Stained cultures were then visualised by inverted light microscopy, transfected cells with β-gal activity appeared blue and were counted.

Since transfection of DNA using Lipofectamine™ has been shown to induce IL-1α release, due to cell death (Komine *et al* 1994), transfections were also performed using polybrene (hexadimethrine bromide) (Sigma). Polybrene induces less cell death and has been a preferred method for transfection of KC in some studies (Jiang *et al* 1991). Two μg pCH110 DNA was mixed with 10 or 40 μg / ml polybrene, and added to cells in 6 well dishes (KC or UMUC-3). After 6 hrs the transfection mix was removed and 10% glycerol added for 3 min at room temperature. Cells were then washed 3 X in PBS and fresh KGM containing additives without EGF was added.

5.3.5 Outgrowth of KC from cutaneous warts

Wart specimens were washed 5 X in PBS and cut into smaller pieces before digestion with trypsin and collagenase as described in section 5.3.1. The suspension was centrifuged at 1800 rpm for 10 min and the pellet resuspended in KGM containing all additives and in some cases with the addition of 600 IU / ml penicillin, 600 μg / ml streptomycin, 250 μg / ml gentamicin and 2.5 μg / ml fungizone. The suspension was cultured in fibronectin coated flasks. Cultures were observed for growth of colonies for up to 20 days after initiation.

5.3.6 Semi-quantitative RT-PCR mRNA analysis

At various time points after transfection/infection with HPV DNA, cells were harvested for mRNA analysis by lysis in 4M guanidinium isothiocyanate and total RNA extraction as described by Chomczynski *et al* (1987). Semi-quantitative RT-

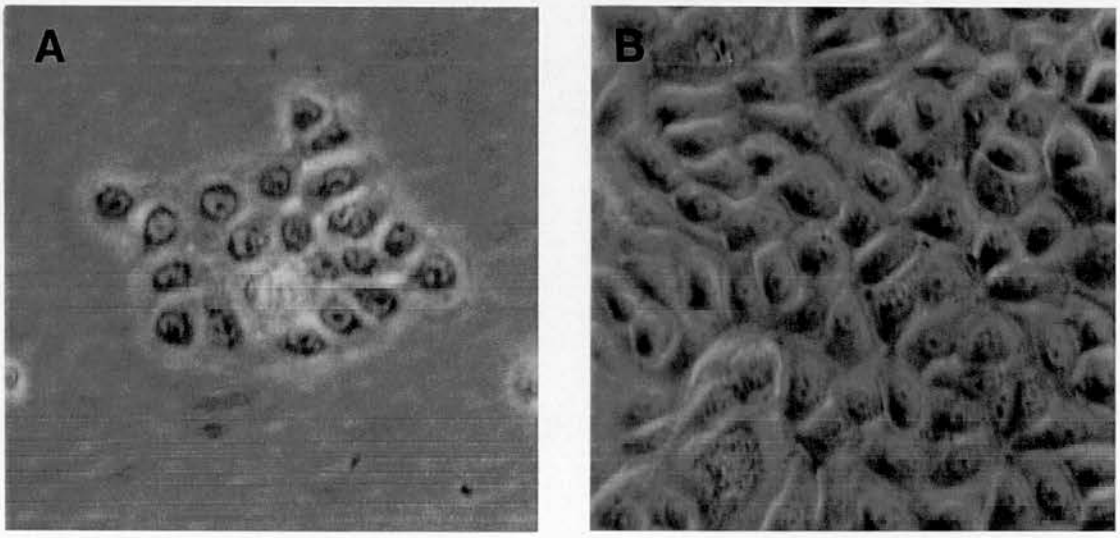
PCR analysis of IL-1α, IL-10, IL-12p40 and IL-8 mRNA was performed using [^{32}P]-labelled primers as described in chapter 4.

5.4 Results

5.4.1 Characterisation of KC

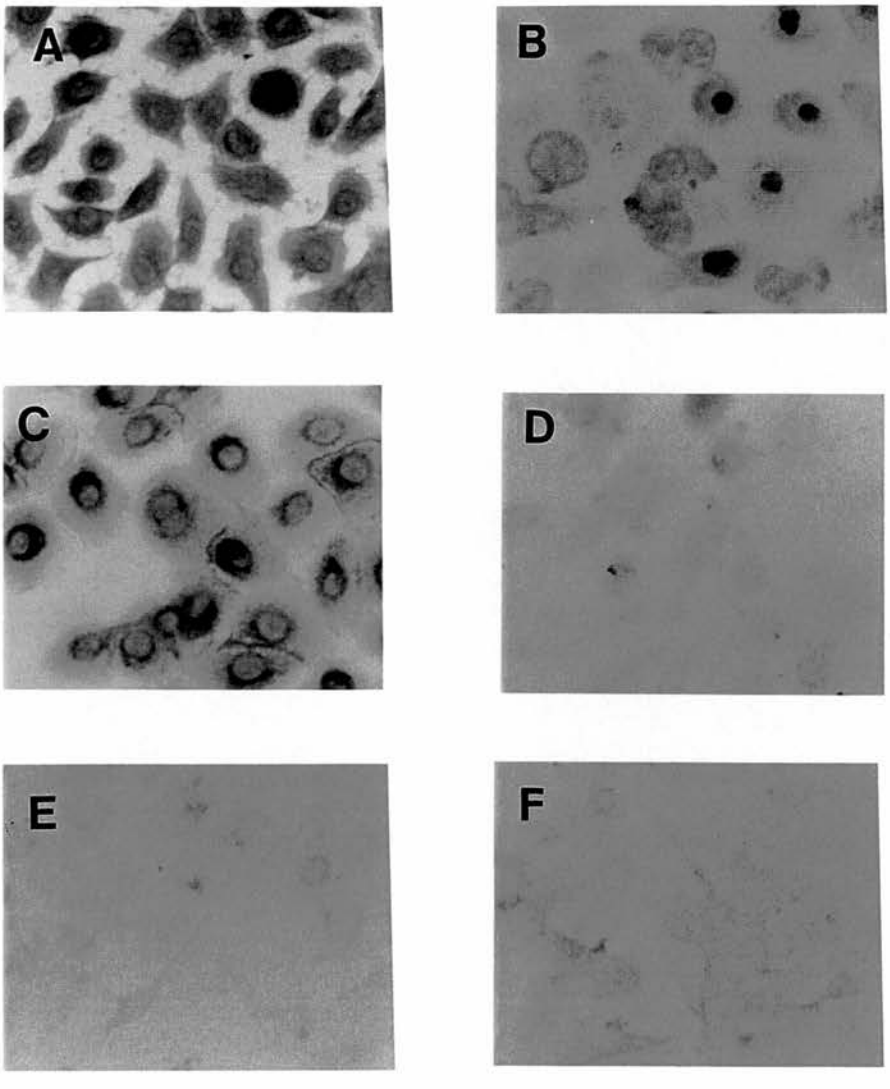
The initiation of cultures from infant foreskins had a 68% success rate. Thirteen percent of cultures became infected before any colonies were established and KC cultures could not be established from 13% of tissue specimens. Six percent of tissue specimens gave rise to cultures which became overtaken with fibroblasts. KC cultures had a doubling time of 24 hrs at optimal growth rate and senesced between passage 3-5. Figure 5.1 shows the initiation of a KC colony after 2 weeks in culture and the morphology of a confluent monolayer of KC. Initially, some KC cultures were phenotyped to characterise the cell population and are shown in Figure 5.2. KC expressed keratin but had lower vimentin expression, a characteristic of epithelial cells. Contaminating fibroblasts typically expressed vimentin but not keratin. KC were MHC class I positive and MHC class II and ICAM-1 negative, as expected for unstimulated KC *in vitro*. The anti-CD3 antibody did not stain the KC and neither did the secondary antibodies.

Figure 5.1 Growth of primary human KC *in vitro*



Photographs of KC culture under phase contrast microscopy. magnification = 100 X. A = initiation of a KC colony after 2 weeks in culture. B = confluent KC after 3 weeks in culture at passage number 1

Figure 5.2 Phenotype of cultured KC

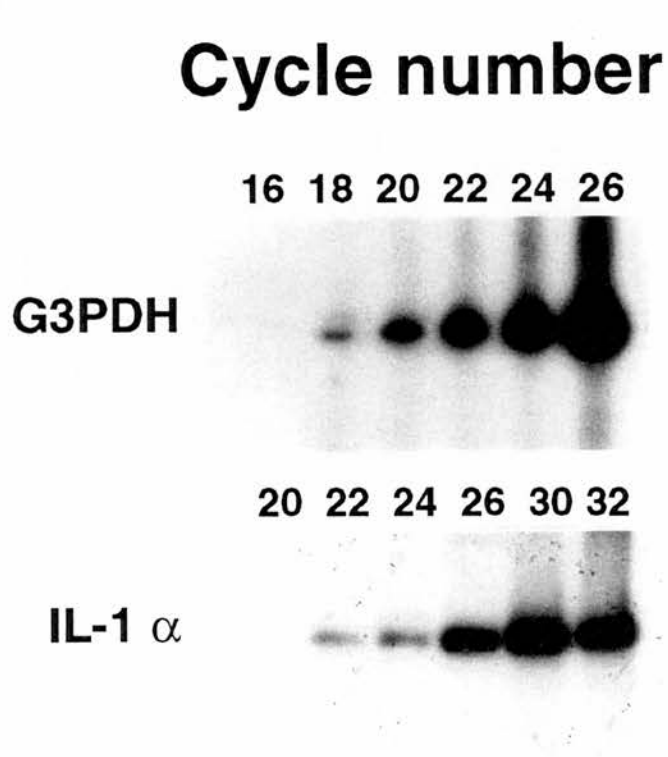


Photomicrographs (magnification = 100 X) of KC cultured on Thermanox™ plastic coverslips and stained using immunoperoxidase for keratin (A), vimentin (B), MHC class I (C), MHC class II (D), ICAM-1 (E) and isotype matched control (F). KC stained with secondary antibodies were negative.

5.4.2 Cytokine mRNA expression in KC infected with HPV

Using the methods described in chapter 4, the optimum cycle number for expression of cytokines in KC were determined and results for IL-1α and G3PDH are shown below.

Figure 5.3 Optimum cycle number determination



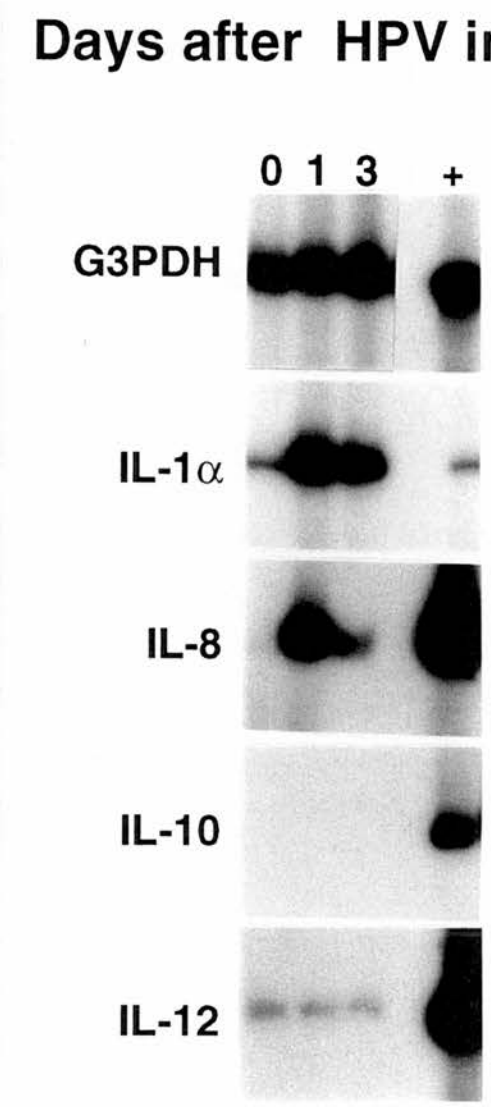
Autoradiographs of amplicons separated by SDS-PAGE, from PCR amplifications at increasing cycle number are shown for IL-1α and G3PDH primers. Optimum cycle numbers were determined as 20 cycles for the G3PDH, 30 for IL-1ra, IL-12p40 and IL-8, 25 for IL-1α, 40 for IL-10.

Cytokine expression in KC cultures infected with HPV isolated from clinical samples are shown at 1 and 3 days after infection in Figure 5.4. Expression of G3PDH mRNA remained constant throughout the experiment whereas IL-1α and IL-8 mRNAs were induced at day 1 after infection with HPV. IL-12p40 mRNA remained at a constant low level throughout the experiment whereas IL-10 mRNA could not be detected at any of the time points. When PCR products were quantified and the cytokine : G3PDH ratios were compared, IL-1α was induced 17 fold on day 1 after infection compared with day 0 samples. IL-8 mRNA was not present in day 0 samples but was induced maximally on day 1, which was 11 fold the amount seen on day 3.

5.4.3 Absence of IL-10 mRNA in human KC in vitro

Stimulated KC were anticipated to be a positive control for expression of IL-10. However when KC were treated with PMA (50 ng / ml), UVB (100 J / m^2, Phillips TL12, broad band UVB source), $NiSO_4$ (200 mg / ml) or SDS (0.1%), no IL-10 transcripts could be detected. This led to a more thorough investigation of the time course and dose dependent induction of IL-10 mRNA expression in KC. Modulation of cytokine expression in KC by UVB irradiation is of particular interest because of the conversion of cutaneous papillomas to squamous cell carcinomas exposed to sunlight in immunosuppressed patients. The results from 4 experiments are shown in Table 5.1 A-D. KC were either exposed to 100 J / m^2 UVB radiation and harvested at 1, 3, 6, 10 and 24 hrs later, or were subjected to increasing doses of UVB from 50-300 J / m^2 and harvested 24 hrs later. Table 5.1 A indicates that IL-10 mRNA could not be detected by Southern blot at any time up to 24 hrs after UVB exposure. However, IL-12p40 mRNA which was not expressed in unstimulated KC was induced maximally 3 hrs after UVB, thus indicating that the cells were responsive to UV treatment.

Figure 5.4 Cytokine mRNA expression in HPV infected KC



Autoradiographs of ^{32}P labelled PCR products of mRNA expression detected by RT-PCR on day 0 (mock infected) days 1 and 3 after infection. Positive controls (+) were as per chapter 4.

Table 5.1A-D Lack of IL-10 in human KC

A	KC UVB irradiated with 100 J / m^2 hrs after UV dose							
	0	1	3	6	10	24	POS	detection
G3PDH	+	+	+	+	+	+	+	EtBr
IL-10	-	-	-	-	-	-	+	Southern
IL-12 p40	-	+	++	-	-	-	+	Southern
TNF-α	+	+/-	+/-	+	+/-	+/-	+	EtBr

B	KC 24 hrs after UVB irradiation UV dose (J / m^2)							
	0	50	75	100	200	300	POS	detection
G3PDH	+	+	+	+	+	+	+	EtBr
IL-10	-	-	-	-	+/-	+	+	Southern
IL-8	+	+	+	++	+	+	+	EtBr
IL-8 (pg / ml)	234	991	1688	1890	1483	935	+	ELISA

C KC 24 hrs after doses of UVB (J / m^2) or IL-1α treatment in low or high calcium.	low calcium 0.06 mM					high calcium 2 mM					
	0	50	75	100	IL-1	0	50	75	100	IL-1	detection
G3PDH	+	+	+	+	+	+	+	+	+	+	EtBr
IL-10	-	-	-	-	-	-	-	-	-	-	Southern
IL-8	-	-	-	+	-	+	+	+	++	+/-	EtBr

D	KC 24 hrs after treatment with $NiSO_4$ (200μg / ml) or SDS (0.1%)				
	0	$NiSO_4$	SDS	POS	detection
G3PDH	+	+	+	+	EtBr
IL-10	-	+/-	+/-	+	silver stain
IL-10	-	-	-	+	Southern
IL-8	-	+/-	+	+	Southern

Non quantitative cytokine mRNA analysis by RT-PCR detected by ethidium bromide staining of agarose gels, Southern blotting and hybridisation with digoxigenin labelled specific riboprobes or silver staining of SDS-PAGE gels. - = not detected, +/- = weak band, + = clearly visible band, ++ = intense band, POS = positive control. No IL-10 protein was detected by ELISA (R and D systems) in any of the supernatants tested. IL-8 protein was detected by ELISA (kindly performed in the laboratory of Dr. J. Ross, University of Edinburgh, Department of Surgery)

TNF-α mRNA expression was present in KC before treatment (Table 5.1 A) but following UVB radiation its expression decreased during 1-3 hrs, and subsequently increased to a maximum at 6 hrs. Table 5.1 B shows IL-8 mRNA and protein expression from KC were both were induced maximally at 100 J / m^2. However, IL-10 mRNA was only induced after exposure to high doses of UVB (200 and 300 J / m^2), but IL-10 protein could not be detected in the supernatants from these cells. No IL-10 protein or mRNA could be detected after exposure to 50 -100 J / m^2 UVB when the calcium concentration in the medium was increased to 2 mM (Table 5.1 C), although IL-8 mRNA was induced to a greater extent after UVB exposure in high calcium medium. IL-10 mRNA was induced after treatment of KC with either irritant or sensitiser chemicals (Table 5.1 D), although it was on the limit of detection since a weak product band was detected by silverstain but was not detected by Southern blot.

5.4.4 KC transfected with pCH110

Results from preliminary studies using transfection with pCH110 showed a low efficiency of transfection in primary human KC (Table 5.2).

Table 5.2 Test transfection of primary human KC

cells	treatment	number of cells with β-gal activity
UMUC-3	2 μg pCH110 + 2 μl Lipofectamine™	10
KC	2 μg pCH110 +1 μl Lipofectamine™	0
KC	2 μg pCH110 + 2 μl Lipofectamine™	1
KC	2 μg pCH110 + 4 μl Lipofectamine™	3
KC	2 μg pCH110 + 8 μl Lipofectamine™	4
KC	8 μl Lipofectamine™	0
KC	no treatment	0

Many cells became unattached and exhibited a change in morphology in cultures treated with 4 and 8 μl of Lipofectamine™. No transfectants were produced in

experiments using 2 μg pCH110 DNA and polybrene (10 or 40 μl) with a 10% glycerol shock.

5.4.5 KC outgrowth from cutaneous warts

No KC were successfully cultured from disaggregated cutaneous warts. One problem was contamination of the tissue in culture, thus requiring higher levels of antibiotic and antimycotic. These high antibiotic levels may have inhibitory effects on KC growth. In addition, digestion of wart specimens with trypsin and collagenase was inefficient at releasing cells from the tissue. The cells which had been released from the wart were often large angular cells which were similar to the terminally differentiated squames seen in normal skin preparations.

5.5 Discussion

5.5.1 Transfection of KC

Primary human KC are typically resistant to DNA transfection (Fenjves, 1994), thus transient expression studies are difficult. Transfection efficiencies of 20 -30 % in KC have been described using liposome formulations of cationic lipopolyamines (Staedel *et al* 1994). Preliminary transfections of pCH110 into primary KC using Lipofectamine™ were of low efficiency. Thorough optimisation of DNA transfection procedures in these primary cultures is necessary before HPV DNA transfections for experimental studies. Important considerations include the observation that transfections using Lipofectamine™ induces cell death and the release of preformed IL-1α from KC (Komine *et al* 1994). The release of IL-1 resulting from the transfection alone is an undesirable feature when investigating cytokine expression in short term cultures as IL-1α may in turn promote the production of other cytokines. Retroviral vectors have been used for DNA delivery to primary human KC with 100 % transduction rates after selection (Garlick *et al* 1991). However, the proviral DNA is integrated into the cellular genome and thus is

less representative of the early stages of a benign HPV infection where DNA remains episomal. Alternatively, replication deficient adenovirus vectors have been used (Setogucho *et al* 1994) and may provide a more efficient way of transfecting KC.

5.5.2 HPV infection of KC

HPV infection of KC resulted in increased levels of expression of IL-1α and IL-8, but not IL-10 or IL-12p40 mRNA. The induction of IL-1α mRNA correlated with *in vivo* data from warts which had increased levels of IL-1α mRNA compared with normal skin (chapter 4) but this is a preliminary observation. Since the preparation of HPV is likely to contain other contaminating proteins, possibly keratins, a CsCl preparation of normal foot skin would make an appropriate control. Further experiments to block the entry of virus, such as with antibodies (Zhou *et al* 1995; Smith *et al* 1993) or by trypsinisation of the KC before infection (Muller *et al* 1995), are needed to demonstrate unequivocally that the increased level of cytokine mRNA expression is due to HPV infection.

5.5.3 Absence of IL-10 production by human KC

IL-10 protein could not be demonstrated in supernatants of human primary KC, either after UVB radiation or the addition of irritant and sensitiser chemicals. IL-10 mRNA was not detected by RT-PCR (40 cycles) at 0, 1, 3, 6, 20 and 24 hrs after 100 J / m^2 UVB exposure. Only when KC were exposed to high doses (200 - 300 J / m^2) of UVB could IL-10 mRNA be detected. The viability of the cells at these high doses were 69% and 34% respectively, whereas KC were >99% viable when lower doses were used. The abundance of IL-10 PCR product was close to the limit of detection since no amplicons could be detected by Southern blot. Despite the lack of IL-10 protein in KC supernatants after UVB radiation, both IL-8 mRNA and protein were detected. A 4.5 fold induction of IL-8 mRNA and an 8 fold induction of IL-8 protein was observed 24 hrs after 100 J / m^2 UVB irradiation, indicating that the KC were responsive to the UVB stimulus. IL-8 mRNA was also observed after

increasing doses of UVB and in cultures containing high and low calcium. IL-12 p40 mRNA was also induced after 1-3 hrs post UVB radiation with 100 J / m^2. Since the weak induction of IL-10 mRNA was not accompanied by a similar increase in detectable IL-10 protein it is possible that the protein was masked by a soluble receptor. Alternatively, the levels of IL-10 protein could be below the sensitivity of the ELISA.

In studies on normal murine skin, Enk *et al* (1992) found IL-10 mRNA and protein after treatment with irritant chemicals or contact sensitisers. IL-10 has been proposed to have a role in the initiation of contact sensitivity in mice (reviewed by Enk, 1994). IL-10 is also induced after exposure of murine KC to UVB radiation, and is involved in the systemic immunosuppression of DTH responses caused by UVB exposure (Rivas *et al* 1992).

In agreement with this study, IL-10 mRNA was not found in human KC either 1-72 hrs after exposure to 15 - 200 J / m^2 UVB radiation or after exposure to PMA, γ-IFN, TNF-α, IL-1-α or $NiSO_4$ (Teunissen *et al* 1995). Similarly Ried *et al* (1994) could not detect IL-10 mRNA between 3-24 hrs after treatment of KC with either IL-1β, IL-6, IL-8, TNF-α or exposure to 10 and 30 mJ / cm^2 UVB. However, Grewe *et al* (1995) have shown IL-10 production by human KC *in vitro* after exposure to UVA and UVB. Using a more sensitive ion-exchange chromatography method, Grewe *et al* (1995) detected maximal IL-10 mRNA at 18 hrs after exposure to 100 J / m^2 UVB, while the levels of protein peaked at 24 hrs. In contrast to data presented here, 70 pg / ml IL-10 protein was found after UVB exposure which increased to 120 pg / ml when the calcium in culture medium was increased to 2 mM.

Enk *et al* (1995) showed that IL-10 mRNA was both constitutively expressed by some human KC lines *in vitro* and up-regulated 24 hrs after exposure to 200 J / m^2 UVB. Although the same primer sequences were used as in this study, Enk *et al*

(1995) used poly A mRNA, a highly purified source of starting material for cDNA synthesis. IL-10 protein was detected (19-34 pg / ml), but the supernatants were concentrated 10 X before assay by ELISA.

The absence of significant levels of IL-10 protein detected in this present study, indicates either that IL-10 mRNA remains untranslated in KC, or levels of protein remain below the sensitivity of the assay (1.5 pg / ml). Either interpretation suggests differential regulation of IL-10 expression between human and murine KC. The data presented here may reflect differences in cytokine repertoire between murine and human KC, as has been shown for IL-3 (Kondo *et al* 1995).

5.6 Conclusion

These preliminary experiments suggest that IL-1α and IL-8 mRNA expression are modulated by HPV *in vitro,* although this requires further confirmation. An interesting observation from this *in vitro* study is that IL-10 protein secretion was not found in human KC, and its mRNA was only detected after high doses of UVB exposure. This finding has implications for the understanding the host response to infections of epithelial surfaces, particularly in sites exposed to sunlight.

Summary and Further Work

Proliferative T cell responses to purified HPV found in some individuals suggest that systemic cell mediated immunity towards HPV occurs *in vivo*. However, clonal populations of HPV-specific T cells could not be isolated from peripheral blood of these subjects. Evidence for the modulation of local immunity was found using immunohistochemical analysis of wart lesions. The density of epidermal LC was significantly reduced in warts compared with normal skin. Increased vascular expression of ICAM-1 and E-selectin was detected in cutaneous warts and may partly account for the increased numbers of memory T cells in the dermis of warts compared with normal skin. However, few intra-epidermal T cells were found in cutaneous warts. ICAM-1 expression on KC could not be found in normal skin or wart epidermis.

In a frozen section adhesion assay designed to model T cell trafficking *in vivo*, fewer activated T cells bound to sections of warts and normal skin than to an inflammatory skin disease such as psoriasis. This may suggest that although there are histological changes associated with HPV infection in warts, there is not a significant increase in adhesive ligands available to promote epidermal trafficking. Taken together these findings suggested a defect in the trafficking of immune effector cells to the epidermis in cutaneous HPV infection.

Preliminary non-quantitative RT-PCR analysis found a reproducible increase in the numbers of wart samples expressing IL-1α mRNA and indicated changes in numbers of warts and normal skin samples expressing IL-8, IL-12 and IL-10 mRNA. There was no apparent difference in the numbers of samples expressing TGF-β, TNF-α, GM-CSF or IL-1β between warts and normal skin. IL-4 and γ-IFN were not detected in either skin type. Semi-quantitative RT-PCR analysis showed a

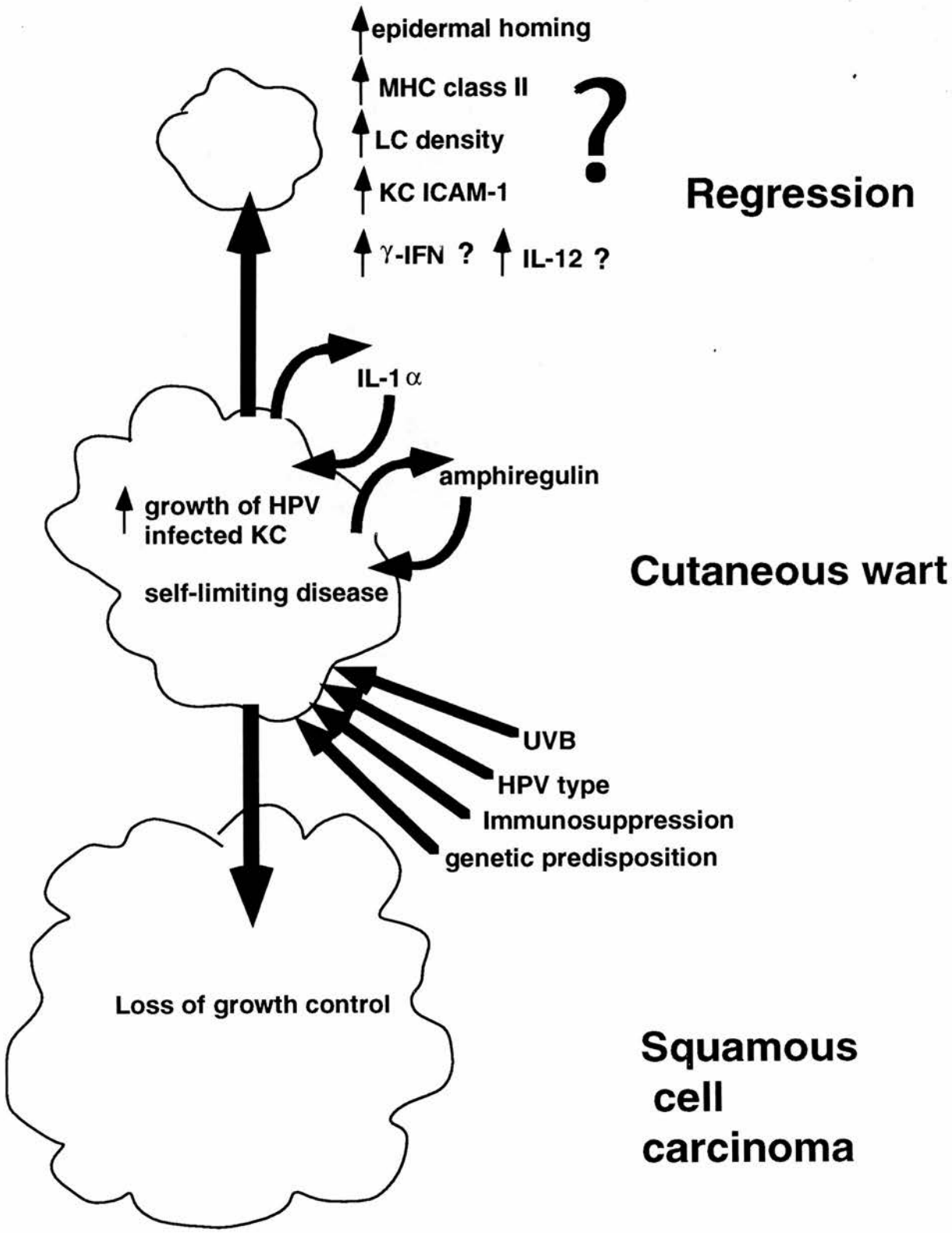
significant increase in IL-1α mRNA expression in warts compared with normal skin, and a decrease in IL-10 mRNA expression in warts compared with normal skin.

Woodworth *et al* (1995) found that IL-1α promoted the growth of KC containing HPV *in vitro* and its action was mediated by AR. Although this phenomenon could be associated with cellular transformation it may also play a role in the self-limiting hyperproliferation observed in benign cutaneous warts. Semi-quantitative RT-PCR analysis showed that warts which expressed IL-1α mRNA also expressed AR and there was a significant correlation between the expression of these cytokines. Although a causal relation ship between IL-1α and AR was not demonstrated in this study, the results indicate that the IL-1α/AR growth promoting autocrine loop may contribute to the epidermal hyperproliferation observed in cutaneous warts (represented diagrammatically in Figure 6.1).

The presence of additional co-factors such as UVB exposure, immunosuppression, and genetic predisposition in conjunction with the type of HPV may promote the cellular transformation and development of squamous cell carcinoma. Without the presence of these "risk factors" the majority of HPV infections in the immunocompetent host will regress. Some features of plane wart and genital wart regression are known, but it is not clear what initiates this regression. The study of IL-1α and AR protein expression in cutaneous warts is required to clarify the potential role of these cytokines in wart growth and persistence

The increase in IL-1α mRNA expression in cutaneous warts was reproduced in initial experiments in which KC monolayers were infected with HPV. Further study is needed to determine if this effect is due to HPV infection alone and not a contaminant of the HPV preparation.

Figure 6.1 A possible mechanism of progression and regression of warts



It would also be interesting to determine whether AR is upregulated in HPV infected cells *in vitro*. In addition other KC growth factors such as transforming growth factor-α and EGF may also be up-regulated in HPV infection and may play a role in promotion of HPV infected KC. Optimisation of KC transfection should provide another method to dissect the effects of HPV on cytokine expression. Experiments using organotypic raft cultures to mimic HPV infection would provide an ideal *in vitro* system to investigate the effects of HPV on cytokine expression either by RT-PCR, *in situ* hybridisation or immunohistochemistry.

Finally, the role of cytokines and KC growth factors, such as AR, in the progression of benign HPV infections to carcinoma, could be determined by analysis of cytokine mRNA expression in specimens of squamous cell carcinomas containing HPV. RT-PCR analysis of cytokine expression in regressing cutaneous warts would also provide valuable information regarding the role of cytokines and growth factors in controlling the duration of the infection and regression.

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Publications and abstracts arising from this work

1 Cytokine mRNA expression in cutaneous warts: induction of interleukin-1α. M.Jackson, R.C.McKenzie, E.C.Benton, J.A.A.Hunter, M.Norval. submitted to Archives of Dermatological Research (1996).

2 Lack of induction of IL-10 expression in human keratinocytes. M.Jackson, K.Thompson, R.Laker, M.Norval, J.A.A.Hunter, R.C.McKenzie. Journal of Investigative Dermatology (1996 in press).

3 Cytokine mRNAs in cutaneous warts (Abstract). M.Jackson, E.C.Benton, M.Norval. 9th International Congress of Immunology. San Francisco July 1995. and 14th International papillomavirus conference. Quebec. July 1995.

4 Cytokine production by human keratinocytes (Abstract). N.R.Hunter, M.Jackson, M.P.Wadhwa, M.C.Carter-McCann. British blood transfusion Society 13th annual scientific meeting. Sterling. September 1995. Transfusion Medicine. **5** S1 p54. 1995.

5 Local immune responses in cutaneous warts: an immunocytochemical study of Langerhans' cells, T cells and adhesion molecules. M.Jackson, E.C.Benton, J.A.A.Hunter, M.Norval. European Journal of Dermatology. 1994 **4** p399-404.

6 Adherence of activated T cells to cutaneous warts. (Abstract). M.Jackson, T.Herremans, E.C.Benton, S.Keohane, M.Norval. 13th International Papillomavirus Conference. October 1994.

7 Local immune responses in cutaneous warts. M.Jackson, E.C.Benton. J.A.A Hunter, M.Norval. 12th European Immunology Meeting. Barcelona. June 1994.

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Cytokine mRNA expression in cutaneous warts: induction of interleukin-1 α

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Introduction

Thirty percent of cutaneous warts caused by human papillomavirus (HPV) infection regress spontaneously within 6 months [4]. Others may persist for years, prove recalcitrant to treatment and may recur. Persistence of infection in an immunocompetent host suggests evasion of local cutaneous immune surveillance. Cell mediated immunity is important in the regression of plane warts [24], genital warts [8] and Shope rabbit papillomas [20]. Previous histological studies have examined phenotypic changes in warts compared with normal skin. A reduced density of Langerhans' cells in the epidermis of non-regressing cutaneous [6,13,28], and mucosal [12,25,28] warts was found. Increased expression of intercellular adhesion molecule -1 (ICAM-1) and E-selectin on vascular endothelial cells and an increased infiltrate of memory T cells were observed in the dermis underlying cutaneous warts compared with normal skin. However, few T cells were detected in the lesional epidermis [13]. These observations suggest a defect in cell trafficking to the site of the HPV infection in the epidermis.

Altered cytokine expression in the skin may be responsible for the absence of immune effector cells at the site of infection. *In vitro* epidermal keratinocytes (KC) are capable of synthesising many cytokines, although there is very little secretion in normal skin. Epidermal Langerhans' cells, dermal fibroblasts, and infiltrating cells also secrete and respond to cytokines. Previous studies indicate that infection with certain HPV types may modulate tumour necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) expression *in vivo* [1,18] and *in vitro* [3,19,31]. Reduced secretion of interleukin-1 (IL-1) α, β , IL-1 receptor antagonist (IL-1ra), IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF), TNF-α and TGF-β in HPV-16/18 immortalised KC cell lines has been observed [31]. Whether these changes are due to transformation or a direct consequence of HPV-16 infection is not clear. It is also uncertain whether these changes in cytokine expression occur in infections with cutaneous HPV types. It is possible that HPV infection may lead to alterations in the cutaneous cytokine network, thus preventing the influx of T cells and other immune effector cells to the local lesion.

In thepresent study we have examined the expression of the mRNAs of IL-1α, IL-1β, intracellular IL-1ra (icIL-1ra), IL-10, IL-12 (p40), IL-8, GM-CSF, TNF-α, TGF-β, IL-4 and γ-interferon (γ-IFN) by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis in cutaneous warts. IL-1α mRNA was significantly up-regulated and IL-10 mRNA

on nylon membrane (Zetaprobe GT Biorad). To confirm the identity of the PCR products, Southern blots were hybridised with specific digoxigenin-labelled riboprobes. The cDNA for each cytokine was cloned into Bluescript II plasmid and riboprobes were generated using the digoxigenin RNA labelling kit (Boehringer Mannheim, Germany). Plasmids containing cDNAs for the cytokines investigated were provided by : Dr. A. Singh, Genentech, CA. USA (TGF-β and TNF-α), Dr. P. Lomedico, Hoffman-La Roche, NJ, USA.(IL-1α), Dr. S. Wolf, Genetics Institute, MA, USA (IL-12 p40), Dr. G. Wong, Genetics Institute, MA, USA (GM-CSF), Dr. J. Oppenheim, National Cancer Institute, MD. USA (IL-8) and IL-10 cDNA was cloned and sequenced from a PCR product using a TA cloning kit (Invitrogen, San Diego, CA, USA). Digoxigenin was detected with anti-digoxigenin alkaline phosphatase antibody (Boehringer) and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. The positive control for cytokine expression was RNA extracted from peripheral blood mononuclear cells (PBMC) stimulated with phytohaemagglutinin (1 μg/ml) and phorbol myristate acetate (PMA) (50 ng/ml) for 24 hrs, except in the case of IL-10, IL-12 p40 and AR where 4 hr stimulated PBMC, a bladder carcinoma cell line (SD), and PMA treated MCF-7 cells were used respectively. Negative controls consisted of RNA prepared without the reverse transcriptase (RT) enzyme in the cDNA synthesis reaction, and PCR reactions with water instead of RT reaction.

Semi-quantitative PCR

Primers were ^{32}P end-labelled with $\gamma[^{32}P]$ -dATP (7000 Ci / mmol. ICN Biochemicals, Thame, UK) using polynucleotide kinase (New England Biolabs, Beverly, MA, USA). PCR using radiolabelled primers was performed as above. Optimum cycle numbers were determined for each primer pair which were in the linear part of the curve of product accumulation versus cycle number. The housekeeping gene, G3PDH, was used to control for differences in abundance of cDNA in RT reactions [26]. Radioactive PCR products were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by exposure of the gels to Kodak X Omat AR autoradiography film, as described previously [14]. Autoradiographs were visualised on the Seescan system image analyser (Seescan Plc, Cambridge, UK) and integrated OD units were obtained using gel analysis software v1.0 1D (Seescan). Ratios of integrated OD units for each cytokine to G3PDH were used to compare the relative abundance of cytokine mRNA between warts and normal skin and were subject to statistical analysis. For each cytokine, all warts, normal skin samples and controls were amplified within the same run, separated on gels and autoradiographed simultaneously. All PCR

quantitative RT-PCR for IL-1α, IL-10, IL-8, IL-12 and icIL-1ra mRNA are shown in Fig. 1. In the normal skin samples IL-1α mRNA was either absent or present at very low levels, whereas 33% warts expressed this cytokine at high levels, and others not at all. In the majority of warts expressing IL-1α mRNA, icIL-1ra mRNA was also detected, and most normal skin samples expressed icIL-1ra mRNA. IL-12 p40 mRNA was found at varying levels in both warts and normal skin with no consistent difference between these two. Confirming preliminary experiments, IL-10 mRNA was shown to be present at low levels in all normal skin samples, whereas in many warts this cytokine was not detected. IL-8 mRNA was found in only one normal skin sample and 5 warts, although in 3 warts it was at barely detectable levels.

Fig. 2 shows cytokine mRNA expression relative to G3PDH expression in warts compared with normal skin samples. Many warts have increased mRNA for IL-1α, icIL-1ra and IL-12 p40 compared with normal skin. However, only IL-1α expression is significantly greater in warts than normal skin ($p = 0.002$). IL-10 mRNA is significantly greater in normal skin than cutaneous warts ($p < 0.05$). Although HPV DNA typing was not included in this study, the location and clinical type of the warts was known. Therefore to determine whether variations in cytokine mRNA expression were dependant on wart location and morphology, IL-1α expression in common hand warts was compared with that in normal skin and plantar warts (Fig. 3). Common hand warts had significantly greater IL-1α mRNA ($p = 0.019$) expression than normal skin, while plantar warts had a significantly greater expression than common warts ($p = 0.003$). This difference in cytokine mRNA expression between warts at different sites was only observed for the expression of IL-1α mRNA. Warts which were positive for IL-1α mRNA were also positive for icIL-1ra mRNA. A positive correlation was found between the expression of IL-1α and icIL-1ra mRNAs in plantar warts (Pearson correlation $r = 0.846$, $p < 0.05$; data not shown). There was no significant correlation between the abundance of IL-1α and icIL-1ra mRNA in either normal skin or common warts. Warts were grouped into high, medium and negative for IL-1α expression. The expression of AR mRNA in cutaneous warts was determined and the autoradiographs are shown in Fig. 4. Warts which were negative for IL-1α were also negative for AR mRNA expression. The expression of AR mRNA was positively correlated with IL-1α mRNA expression in warts (Pearson correlation $r = 0.590$, $p < 0.05$; data not shown). No such correlation was observed in normal skin.

KC but is inhibitory to the growth of normal KC *in vitro* [31]. This induction of proliferation is mediated by autocrine AR expression. Although a causal relationship cannot be assumed from mRNA expression in cutaneous warts, one speculation is that IL-1α may induce the observed AR mRNA expression in cutaneous warts and thus promote the growth of HPV-infected KC. An increase in EGF receptor mRNA expression in condyloma has also been observed [29]. In addition, HPV-16 E5 interacts with the EGF receptor when expressed on fibroblasts *in vitro* [21]. This interaction renders the cells more sensitive to exogenous EGF, and thus increases their proliferative activity. This phenomenon could explain the hyper-proliferative nature of epidermal KC in cutaneous warts.

If IL-1α is not released from KC, it is possible that it could act in an intracellular autocrine way, by signalling receptors from within the cell. Intracellular autocrine action has been described for several cytokines including GM-CSF, IL-6, IL-3 and platelet derived growth factor in immortalised cells [17].

Up-regulated IL-10 expression has been shown to contribute to the persistence of parasitic and retroviral infections [23]. In this study IL-10 mRNA is expressed in 9/11 normal skin samples at low levels in the majority. However, IL-10 mRNA expression was significantly lower in cutaneous warts compared with normal skin. Thus, HPV persistence in cutaneous warts is not due to up-regulated IL-10 mRNA expression.

Preliminary non-quantitative experiments found no qualitative differences between TNF-α, TGF-β, IL-1β or GM-CSF mRNA expression in warts and normal skin. IL-4 or γ-IFN amplicons could not be detected by agarose gel electrophoresis. Although expression of these cytokines has not been investigated previously in cutaneous warts, TGF-β and TNF-α mRNAs are induced in epidermodysplasia verruciformis (EV) lesions [18]. A decrease in TGF-β mRNA expression in HPV 6/11 condylomata was observed compared with normal tissue [1], and no change in TGF-β mRNA expression was seen in HPV 16/18 immortalised cell lines *in vitro* [3]. The induction of TGF-β mRNA reported in EV lesions was more marked in samples showing signs of malignancy. This difference may therefore be associated with progression to malignancy. TNF-α mRNA is expressed in HPV-16 tumourigenic cell lines [19]. In contrast Woodworth and Simpson [31] found a reduced secretion of TNF-α in HPV16/18 immortalised cell lines *in vitro*. Discrepancy in these *in vitro* results may be associated with differences between tumourigenic cell lines and non-tumourigenic cell lines.

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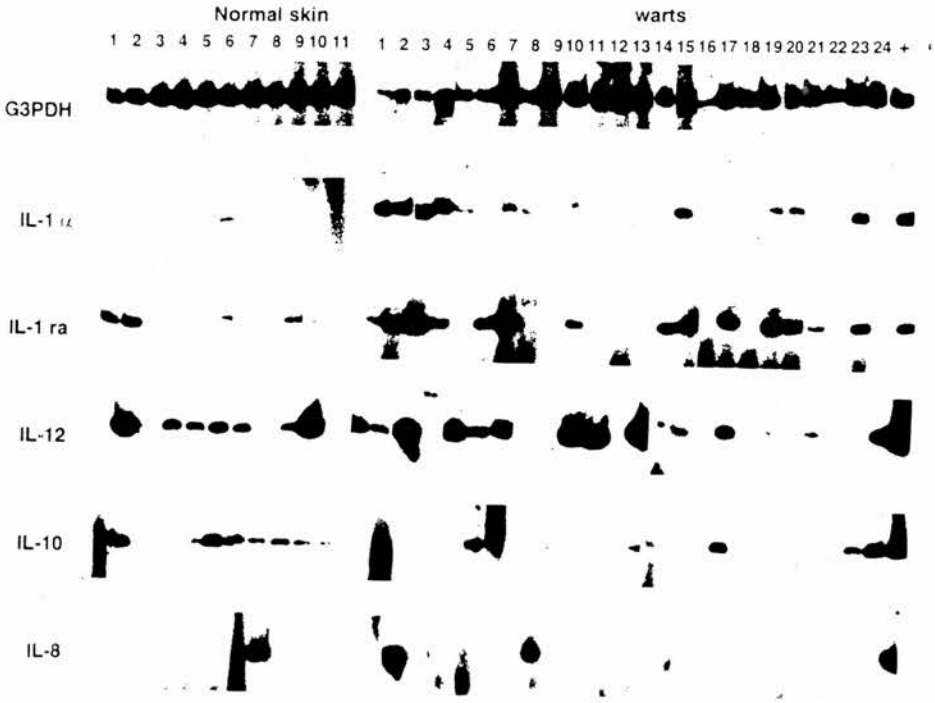
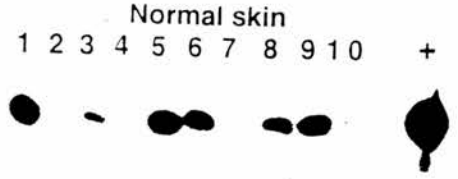
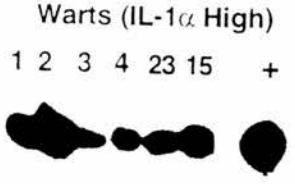
Table 1

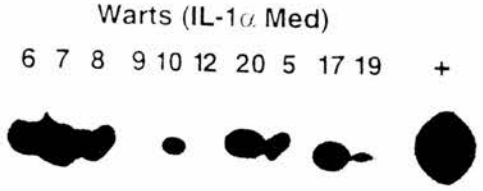
Cytokine		Primer sequence	Product size (bp)	Source
IL-1α	5' =	5' ATGGCCAAAGTTCCAGACATGTTT3'	816	Clontech
	3' =	5' GGTTTTCCAGTATCTGAAAGTCAG3'		
IL-1β	5' =	5'AAACAGATGAAGTGCTCCTTCCAG3'	388	[15]
	3' =	5'TGGAGAACACCACTTGTTGCTCCA		
TNF-α	5' =	5'ATGAGCACTGAAAGCATGATCCGG3'	695	[15]
	3' =	5'GCAATGATCCCAAAGTAGACCTGCCC3'		
TGF-β	5' =	5'CGCCTTAGCGCCCACTGCTCCTGTGT3'	533	[27]
	3' =	5'GGGGCGGGACCTCAGCTGCACTTG3'		
IL-4	5' =	5'ATGGGTCTCACCTCCCAACTGCT3'	456	Clontech
	3' =	5'CGAACACTTTGAATATTTCTCTCTCAT3'		
γ –IFN	5' =	5'GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC3'	427	Clontech
	3' =	5'CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG3'		
GMCSF	5' =	5'ATGTGGCTGCAGAGCCTGCTGC3'	424	[16]
	3' =	5'CTGGCTCCCAGCAGTCAAAGGG3'		
IL-8	5' =	5'ATGACTTCCAAGCTGGCCGTGGCT 3',	289	[15]
	3' =	5' TCTCAGCCCTCTTCAAAAACTTCTC 3'		
IL-10	5' =	5' AAGCTGAGAACCAAGACCCAGACATCAAGGCG 3'	328	Clontech
	3' =	5'AGCTATCCCAGAGCCCCAGATCCGATTTTGG 3'		
IL-12 p40	5'=	5' CTACTTCTCCCTGACATTCT 3'	269	[32]
	3'=	5' TGGTCTATTCCGTTGTGTC 3'		
icIL-1 ra	5' =	5' CAGAAGACCTCCTGTCCTATGAGG 3'	470	[5]
	3' =	5' TTGTCAGGCATATTGGTGAGGCTGAC 3'		
G3PDH	5' =	5' CATGTGGGCCATGAGGTCCACCAC 3'	983	Clontech
	3' =	5' TGAAGGTCGGAGTCAACGGATTTGGT 3'		
AR	5' = TCCTCGGGAGCCGACTATGAC 3'		329	[10]
	3' = GGACTTTTCCCCACACCG 3'			

PCR was performed using the above primer pairs which spanned at least one intron to distinguish cDNA product from genomic DNA.

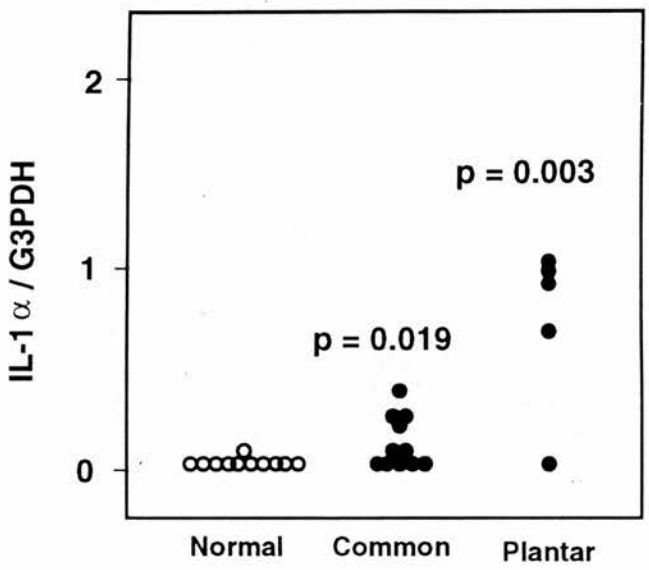

Normal skin
warts
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 +
G3PDH
IL-1 α
IL-1 ra
IL-12
IL-10
IL-8


Normal skin
1 2 3 4 5 6 7 8 9 1 0 +


Warts (IL-1α High)
1 2 3 4 23 15 +


Warts (IL-1α Med)
6 7 8 9 10 12 20 5 17 19 +


Warts (IL-1α Neg)
1122 14 18 21 +


IL-1 α / G3PDH
2
1
0
p = 0.003
p = 0.019
Normal
Common
Plantar

Lack of IL-10

Induction of IL-10 Expression in Human Keratinocytes

We read with interest the report by Grewe *et al* [4] discussing the induction of IL-10 following ultraviolet B (UV-B) and ultraviolet A1 irradiation of human keratinocytes (KC) in primary culture. We too have performed similar UV-B experiments, and have also treated human KC and A431 epidermoid carcinoma cells with sensitizing and irritant chemicals. We were unable, however, to detect any IL-10 protein in the culture supernatants by an enzyme-linked immunosorbent assay (ELISA) with a sensitivity of 1.5 pg/ml before or after any of the above treatments. Furthermore, we could not detect IL-10 mRNA except 24 h after UV-B doses of 200 and 300 J/m^2. Generally, our results agree with those of Teunissen *et al*, who were unable to detect interleukin 10 (IL-10) mRNA (35 thermal cycles) or IL-10 protein in KC after UV-B (15-200 J/m^2), or treatment with nickel sulfate, IL-1α/β, tumor necrosis factor-α, interferon-γ, lipopolysaccharide, phorbol ester, or supernatants from phytohemagglutinin-stimulated T cells (Teunissen MBM, Koomen CW, de Waal Malefyt R, Bos JD: Human keratinocytes are unable to produce IL-10. (abstract) *J Invest Dermatol* 102:632, 1994).

The experiments that we performed utilized KC derived from either foreskin or abdominal skin from five different donors. The sensitive reverse transcription polymerase chain reaction [5] was employed using ethidium bromide to detect amplified products resolved on 1.5% agarose gels and silver staining with a commercial kit (Bio-Rad laboratories Ltd), or ^{33}P-labeled primers to visualize products on 12.5% polyacrylamide gels. We also carried out southern blotting of the agarose gels and hybridized IL-10 amplicons with a digoxigenin-labeled human IL-10 probe, which was detected with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The primers were obtained from Clontech and successfully detected abundant IL-10 mRNA in RNA derived from lipopolysaccharide- or phorbol-ester-stimulated peripheral blood monocytes.

The KC from four different donors were negative for IL-10 mRNA for up to 24 h following irradiation with 100 J/m^2 of broadband UV-B, (280-320 nm). IL-10 mRNA was detected 24 h after UV-B irradiation with 200 and 300 J/m^2 only; however, we had to extend the thermal cycling to 40 cycles to detect even a weak band. In Grewe's study, 28 cycles were typically used. In contrast to the results presented in Grewe's study, 100 J/m^2 and timepoints earlier than 24 h did not induce IL-10 mRNA. Densitometry of the IL-10 southern blots probed with digoxigenin showed a 24-fold increase in IL-10 mRNA with 300 J/m^2, but no detectable IL-10 protein was found in the culture supernatants.

Although no IL-10 mRNA induction was observed, a 4.5-fold induction of IL-8 mRNA and an 8-fold increase in IL-8 protein (measured by ELISA) was noted 24 h after irradiation with 100 J/m^2 and a 30-fold induction of mRNA for the p40 subunit of IL-12 was seen 3 h after 100 J/m^2. Thus, the cells were clearly responsive to UV-B. Changing the calcium content of the medium from 0.06 mM to 2 mM for 48 h prior to irradiation or stimulating the KC with 10 ng/ml IL-1α, 200 U/ml IL-2, 0.01% sodium dodecyl sulfate, or 200 μg/ml nickel sulfate had no effect on the induction of IL-10 mRNA. Furthermore, treatment of A431 cells with irritants [sodium dodecyl sulfate (0.0001-0.01%) or nonanoic acid (0.0001-0.01%)], sensitizers [nickel sulfate (10-200 μg/ml) or Diphenylcyprone (0.000003-0.003%)], or phorbol ester (10 ng/ml) were all ineffective in inducing IL-10 mRNA or protein. Phorbol ester treatment, however, did induce tumor necrosis factor-α mRNA, and irritants induced IL-8 mRNA showing that the cells were capable of responding to stimulation with cytokine gene expression.

Our observations and those of Teunissen *et al* raise several points. First, if IL-10 mRNA is induced but no protein is detected in the culture supernatant, is the IL-10 protein being sequestered, possibly by a soluble receptor, or is this another case that illustrates the pitfalls of measuring only mRNA abundance? Second, the induction of IL-10 mRNA [1] and protein [2] by sensitizers and IL-1 in murine KC could not be reproduced in human KC, either in our studies or in those of Teunissen *et al*. This suggests that IL-10 may be another example, like IL-3 in which human and murine KC express different cytokines [7]. The induction of IL-10 in murine KC and epidermis by sensitizers but not irritants has been demonstrated both *in vitro* [1,2] and *in vivo* [6]. It has been proposed that this property may be a useful means of distinguishing sensitizers from irritants *in vitro*. Clearly, this test may be inappropriate if human KC do not respond to sensitizers. Finally, is the discrepancy in results between different studies due to differences in the primers or ELISA kits used (Stratagene primers and ELISA kits from Genzyme and Laboserve in [4] and Clontech primers and ELISA kits from R & D systems in our study), or does it represent true phenotypic differences in the expression of IL-10 in human cells? In support of the latter is a recent study [3], which showed that only five out of nine KC cultures from human subjects showed constitutive IL-10 mRNA expression and that culture supernatants had to be concentrated 10-fold before low (<30 pg/ml) levels of IL-10 protein were detected, in four irradiated cultures. These questions require resolution before epidermal IL-10 expression can be effectively integrated into the skin cytokine network in human subjects.

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PANCREATIC BETA CELL-SPECIFIC HUMORAL AUTOIMMUNITY TRIGGERED BY A UNIQUE RETROVIRAL PEPTIDE J.-W. Yoon, H.-C. Liang, and H.-S. Jun. Fac. of Med., Univ. of Calgary, Calgary, Alberta, Canada

IDDM results from destruction of pancreatic beta cells by beta cell-specific autoimmune processes; however, what triggers this beta cell-specific autoimmunity is unknown. We recently found that retrovirus particles are present in only beta cells from 4 deceased, recent-onset IDDM patients, but not in any cells of 10 non-diabetic control subjects.This investigation was initiated to determine if retroviral proteins may trigger beta cell-specific autoimmunity in humans. RNA was extracted from the islets of these 14 individuals and a cDNA library was constructed for each. The conserved retroviral *pol* gene region from the cDNA libraries was amplified with mixed oligonucleotide primer which is the highly conserved sequence of the retroviral reverse transcriptase N-terminal coding region.The amplified fragments were cloned into the pCR™ II vector and sequenced. The sequences were compared to known retroviral sequences on file at the NIH GenBank. Through this analysis, we identified a 48 nucleotide region of the *pol* gene (which encodes a 16 amino acid peptide) in only the islets of the 4 deceased IDDM patients, but not in the islets of the 10 control subjects, nor in any other tissues of either group. We synthesized this IDDM-specific 16 amino acid peptide and found that sera from nearly 70% of 260 new onset IDDM patients reacted with it (titer range: 1:10-1:1280), while less than 1% of sera from 515 non-diabetic control subjects reacted with the peptide (titers less than 1:10). On the basis of the above observations, we conclude that this IDDM-specific retroviral peptide may trigger beta cell-specific autoimmunity, at least at the humoral level.

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IDENTIFICATION OF T-CELL EPITOPES IN VACCINEES WITH HBsAg. M.C. Honorati*, P. Dolzani*, E. Mariani*, A. Piacentini*, L. Cattini* and A. Facchini*§. *Lab. Immunol. Genet., Ist. Codivilla Putti, IOR, Bologna; §Ist. Clin. Med. Gastroenterol. Univ. Bologna, Italy.

In order to identify the immunodominant region of HBsAg involved in the recognition by CD4+ cell, we analyzed the in vitro response of T lymphocytes against whole HBsAg or partial peptidic sequences of this antigen. T lymphocyte clones (TLC) were obtained by limiting dilution assay from PBMC of 10 healthy volunteers immunized with recombinant HBsAg. TLC were CD4+ CD25+ by cytofluorimetric analysis.

Recombinant HBsAg or 10-20 mer synthetic peptides in linear format representing HBsAg residues were used for antigen stimulation in vitro which was evaluated by ^{3}H-thymidine incorporation. The 21-30 sequence was the HBsAg region able to stimulate in vitro TLC from all subjects examined. On the basis of the panel of cytokine produced, antigen specific TLC clones resulted to belong to Th2 subset. We conclude that the sequence 21-30 represents the major immunodominant epitope of HBsAg in vaccined subjects.

This work was supported by grants from P.F. Ministero della Sanità "Protezione dall'infezione da virus dell'epatite B in operatori ospedalieri: risposta immunitaria cellulare ed anticorpale e valutazione della memoria immunologica" and IOR Ricerca Corrente.

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Molecular analysis of cellular immune response in cervical cancer associated to human papillomavirus 16. J.M. Alcocer-Gonzalez, R. Tamez, and V. Madrid-Marina. School of Biological Sciences, University Autonomous of Nuevo Leon and Molecular Virology Division, Instituto Nacional de Salud Publica Cuernavaca, Morelos, Mèxico.

Cervical cancer is a disease with very high frequency, around 500.000 women are affected every year worldwide, and in the developing countries represent 30% of all malignant tumors. Epidemiologic studies suggest that this cancer is related to chemical compounds, smoking, and sexual habits. In the origen and development of this cancer are involved: 1) mutations in some oncogenes (c-myc, ras), 2) tumor supressor genes (p53, Rb), 3) association to human papillomavirus (HPV16, 18, 33), and 4) the immunologic status of the host. Recently, it has been demonstrated that local cellular immune response play an important role in the development of cervical cancer. The aim of this study is to analyze the local gene expression of cytokines and surface molecules that participate in activation of T-cell in biopsies of cervical cancer.

The presence of HPV in biopsies of cervical cancer was done by DNA amplification by PCR, and the HPV typification by restriction analysis of the PCR product. So far, we have analyzed 50 biopsies, 35 were positives to HPV16, indicating that HPV16 is the most prevalent. In this samples we studied the mRNA expression of CD3, CD4, and CD8, the cytokine expression produced by T-lymphocytes, Th1 (IL-2, INF-gamma); Th2 (IL-4, IL-5, and IL-10), as well as inflammatory cytokines (IL-6 and TNF-alpha) by RT-PCR. These tumors show differential expression of mRNA of CD4 and CD8; while 40% express CD4, all of them express CD8. More of 80% did not express IL-2, IL-6 and TNF-alpha; and all express IL-4, INF-gamma and TGF-beta.

Our results indicate a differential expression of cytokines from Th1 and Th2, with a predominant expression of Th2 cytokines, as well as there is not expression of inflammatory cytokines.

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THE FACTORS AFFECTING ANTIBODY DEPENDENT ENHANCEMENT IN DENGUE VIRAL INFECTION Li-Jung Cluen[1], Mei-Jane Wu[1], May C. Chu[1], Dennis W. Trent[2], Chun Chun[1], [1] Institute of Preventive Medicine, National Defense Medical Center, [2] Division of Vector-Borne Infectious Diseases, Center for Disease Prevention and Control

Incidences of dengue fever (DF) and dengue hemorrhagic fever / dengue shock syndrome (DHF/DSS) have increased worldwide. The causes of DHF/DSS are subjects of research interest. There are two major hypotheses have been postulated: (1) it is due to the virulence difference in different virus strain or (2) it is due to pre-existing antibody in the host, i.e. the antibody dependent enhancement (ADE). The ADE theory is based on observations that most reticuloendothelial cells express varying degree of Fc-receptors (FcRs) for immunoglobulins, hence, circulating antibodies combine with infectious viruses and enter the cell via the attachment of the antibodies to the FcRs. However, different population infected with the same serotypes do not always develop equally severe disease, therefore, viral and/or host factors may also play a role in disease pathogenesis. We conduct this study by: (1) collecting peripheral blood leukocytes (PBL) from dengue immune and non-immune donors to infect with dengue viruses in antibody dependent assay and (2) using their sera as antibody source to proceed ADE experiments in cell lines. Experimental results demonstrated that: (1) ADE data on human PBL showed that host variation also played a decision role, (2) the BHK-21 had FcR-I, FcR-II and FcR-III and could demonstrate ADE phenomena, (3) the enhancing titer of Ab and enhancing folds of viral yields varied with virus strain. In general, 16681 (DEN-2 Thailand DHF strain) could produce higher peak viral yields at lower Ab concentration than PL046 (DEN-2 Taiwan DF strain), paralleled with the severity of clinical symptom, and (4) the differences of E region deduced amino acid sequence between PL046 and 16681 might cause the changes of envelope protein secondary structure which will influence the entry of virus and the binding of virus and Ab. It is interesting to figure out whether the original susceptibility of PBL to dengue virus or the enhancing ability or the viral virulence is more important in DHF/DSS pathogenesis.

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RESPIRATORY SYNCYTIAL VIRUS-INDUCED INTERFERON-α INHIBITS CELL IMMUNITY IN VITRO. F.M. Preston, P.L. Beier and J.H. Pope. Sir Albert Sakzewski Virus Research Centre, Herston Rd, Herston, Qld, Australia 4029.

Respiratory syncytial virus (RSV) has been found to suppress T-cell mitogenesis and the proliferative response to RSV. We have previously found that this inhibition was mediated by an extracellular RSV-induced factor.The factor has now been clearly identified as interferon (IFN)-α. The RSV-induced IFN-α bound strongly to the cells and inhibited the anti-RSV response only when added within the first few days of stimulation. The proliferation of primary antigen-specific T-cell lines was not affected by either RSV-induced or purified IFN-α. However, no inhibition of IL-2 production nor cell surface expression of various activation markers was observed. Previous studies have shown that RSV induces an IL-1-inhibitor but we were unable to detect this activity. Inhibition by RSV-induced IFN-α was unrelated to the levels of IL-1, IL-2, IL-6, or IFN-γ induced by RSV in vitro, nor to the presence of TNF-α, prostaglandin or IL-10. Although RSV has been considered a poor inducer of INF in vitro and in vivo, the results of this study clearly suggest a re-examination of the possible role of IFN-α in the pathogenesis of RSV infection and disease.

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CYTOKINE mRNAS IN CUTANEOUS WARTS.
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There are over 70 types of human papillomavirus (HPV), of which HPV-1 causes plantar warts and HPV-2 common mosaic warts on cutaneous sites. Cell mediated immunity (particularly at a local level) plays a major role in determining the duration and regression of HPV infections. Previous studies in our laboratory provided evidence that there is an infiltrate of CD3+ and CD45RO+ T cells in the dermis of cutaneous warts, but this infiltrate is not found in the epidermis. A defect in the normal epidermal cytokine network within cutaneous warts could have a role in inhibiting the trafficking of T cells into the site of the viral infection. The following study aims to investigate the expression of cytokines in HPV lesions using the RT-PCR technique to detect mRNA transcripts in biopsies of warts, and to compare this expression with cytokine mRNA in normal skin biopsies. Constitutive expression of TNFα mRNA was detected in 83% of normal skin biopsies, and was also present in 69% of the warts. ICAM-1 mRNA was found in 83% of normal skin and 50% of wart samples. IL-1α and β mRNAs were not detected in normal skin, but both were upregulated in 62% of the wart lesions. No IL-8, IL-10, GMCSF, IFN γ or IL-4 mRNAs have been found in any samples tested. The IL-1 which is expressed in many of the warts could potentially initiate an inflammatory response, but the up-regulation of IL-1 has not led to the induction of IL-8. If IL-8 is not present, perhaps this may account for the lack of T cells in the epidermis of cutaneous warts and contribute to the frequent persistence of these lesions.

CYTOKINE PRODUCTION BY HUMAN KERATINOCYTES.

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Biological dressings comprising a living layer of keratinocytes supported by a matrix of fibrin sealant has been proposed for the treatment of chronic leg ulcers. However, this technique is both labour intensive and expensive in both production and cryogenic storage of grafts.

An alternative therapy would be to use karatinocyte supernatant which is rich in cell derived growth factors and cytokines to induce native wound healing in such patients.

Here, we have investigated cytokine production in keratinocytes at different stages of differentiation.

Using RT-PCR technology to detect cytokine mRNA for IL-1 and ß and TGF and ß, we examined three different keratinocyte lines over a period of one week. Differentiation was induced by the addition of Ca^{++}.

TABLE 1:

Cell Line K290	CYTOKINE PCR PRODUCT DETECTED				
Conditions	IL-1	IL-1ß	TGF	TGFß	ICAM-1
undifferentiated 24 hrs	+	+	+	+	-
post differentiated 24 hrs	+	+	+	+	-
" " 96hrs	+	+	+	+	-
" " 144hrs	+	+	+	+	-

Our results show that the mRNA message is present in all samples irrespective of their differentiation status. Similar results were observed in the three different cell lines examined.

To conclude, our data implies that keratinocytes produce supernatants containing growth enhancing cytokines which if combined with a fibrin sealant matrix could be used in the treatment of chronic leg ulcers. Additionally, these data could be used as a template for formulating a synthetic mixture of cytokines.

Investigative report
Eur J Dermatol 1994; 4: 399-404

Local immune responses in cutaneous warts : an immunocytochemical study of Langerhans' cells, T cells and adhesion molecules

MELANY JACKSON, E. CLAIRE BENTON, JOHN A.A. HUNTER, MARY NORVAL

Cutaneous warts, caused by infection with human papillomavirus (HPV) frequently persist for months or years; it is not clear what aspects of the immune system are most important in their control and resolution. The present study concentrated on an immunocytochemical analysis of Langerhans' cells and T cells in cutaneous warts together with an assessment of adhesion molecule expression (ICAM-1 and ELAM-1). Comparisons were made throughout with skin from normal subjects and from patients with psoriasis. There was an increase in numbers of $CD3^+$ (pan T), $CD45RO^+$ (memory T) cells in the dermis of warts compared with normal skin. In addition ICAM-1 and ELAM-1 expression were increased in the dermis of warts compared with normal skin which may be responsible for the influx of T cells into this site. However, a large reduction of $CD3^+$ $CD45RO^+$ T cells was seen in the epidermis of warts compared with normal skin, and epidermal ICAM-1 was not observed in warts. In 75% of the warts studied epidermal Langerhans' cell numbers were reduced in comparison with normal or psoriatic skin. Thus, a decrease in Langerhans' cells and a lack of adhesion molecule expression in the epidermis of warts may lead to a reduced influx of T cells into the lesion itself, although T cells are evidently present in the dermis underlying the infected site. It is possible that these local effects are induced by factors produced from keratinocytes as a result of HPV infection.

The role of acquired immunity in the control and regression of cutaneous warts remains unclear. Clinical observations suggest that cell mediated immunity (CMI) is more important than the humoral response. Thus, patients with depressed CMI caused by tumours, drugs or HIV infections show an increased prevalence of human papillomavirus (HPV) infection [1, 2], whereas patients with defective antibody production do not generally develop extensive or persistent HPV infections. Experimentally it has proved difficult to measure the cell mediated immune response towards HPV: for example, individuals with warts showed little response to purified HPV 1 or 2 viral antigens in *in vitro* lymphoproliferation assays, and no correlation between lymphocyte proliferative response and the clinical course of the wart has been found [3].

Although *in vitro* evidence for a systemic cellular immune response is poor, there is however, histological evidence to support a local role for cellular immunity in warts. In non-regressing warts a decrease in the density of epidermal Langerhans' cells (LC) has been reported in some cutaneous [3-5] and mucosal [5-7] HPV lesions and was more marked in the lesions of immunosuppressed organ allograft recipients [5]. This decrease of LC could contribute to the non-responsiveness of these patients to HPV as LC are the major antigen presenting cell of the skin. Moreover, Chardonnet *et al.* found an increase of LC in the epidermis of a regressing wart together

with an intense mononuclear infiltrate into the dermis [4]. In regressing plane warts, an infiltrate of T and mononuclear cells was found adjacent to damaged keratinocytes [8]. However, T cell infiltration of dermis has also been observed in non-regressing cutaneous warts [4, 9] and mucosal lesions [9, 10] and might be due to changes in adhesion molecule expression within the HPV lesion, which could alter the trafficking of immunologically important cells to the wart.

Inter-cellular adhesion molecule 1 (ICAM-1) is constitiutively expressed on vascular endothelial cells and is upregulated on these cells and induced on epidermal keratinocytes in psoriasis and other inflammatory reactions of the skin [11-14]. The expression of ICAM-1 on vascular endothelial cells promotes strong attachment of LFA-1$^+$ T cells and their subsequent egression into the tissues. ICAM-1 has also been suggested as a mechanism of T cell binding to keratinocytes *in vitro* and is a likely candidate for epidermotropism of T cells *in vivo*.

Endothelial leukocyte adhesion molecule-1 (ELAM-1, E-Selectin), not normally expressed on human skin is induced on the surface of activated endothelial cells [15] and has been implicated in the binding of polymorphonuclear cells (PMN) *in vitro* [16]. It has now been demonstrated that a subset of memory cells bind to ELAM-1 *in vitro* [17]. These cells are characterised by high expression of CD58 (LFA-3) and CD45RO together with the expression of cutaneous lymphocyte associated antigen (CLA). ELAM-1 has been implicated in the homing of these T cells into the skin [17, 18].

To clarify further the immune response in cutaneous warts, expression of ELAM-1 and ICAM-1 has been examined in HPV-induced lesions. In addition, infiltration of T cells (CD3) and the memory T cell phenotype (CD45RO) in the dermis and epidermis has been studied. Finally, the density of LC in the epidermis of these lesions has been measured. A comparison was made with normal skin and lesional skin from chronic plaque psoriasis.

Methods

Viral warts were obtained from 8 patients aged 17-55 years. Five were papilliferous facial warts which had been present for less than 1 year (numbers 1-5). The remaining three were a plantar wart (number 6) and two hand warts (numbers 7 + 8) all of which had persisted for at least two years. None of the warts had been treated prior to their removal by curettage. All specimens were bisected, half being formalin-fixed for routine histological examination, the remainder being snap frozen in liquid nitrogen and stored at - 70° C until processing. Control specimens consisted of 6 mm punch biopsies from normal forearm skin of six volunteers (age 20-57 years) and from involved skin of four psoriatic patients (age 21-46 years). Five µm cryostat sections were cut and air dried overnight before fixing in acetone for 20 min at room temperature. Slides were air dried and stored at - 20° C until used. Indirect immunoperoxidase staining was performed using the antibodies listed in *Table I*.

Sections were immersed in tris-buffered saline (TBS) pH 7.6, and endogenous peroxidase blocked with 0.6% peroxide in ethanol for 5 min, followed by 20% rabbit serum in TBS for 20 min. Sections were washed three times in TBS after each step. Primary antibodies, diluted as shown in *Table I*, in 10% human serum in TBS, were added to the slides and they were incubated in a humid chamber at 4° C overnight. The human serum used throughout was from the same pooled batch which had been heat inactivated. Ten % human serum in TBS was used as a control and additional controls contained irrelevant monoclonal antibodies of the same isotypes as those used in *Table I*. The slides were washed in TBS and peroxidase conjugate (Dako P161), diluted 1/100 in 10 % human serum in TBS, was layered on top of the slides which were incubated for 30 min at room temperature. The developing substrate was one 10 mg diaminobenzidine tetrahydrochloride tablet (Sigma D-5905) dissolved in 15 ml TBS containing 12 µl 30% hydrogen peroxide solution filtered through a 0.2 µm filter. Slides were lightly counterstained with haematoxylin to enable visualisation of the dermis and epidermis before dehydrating the slides through 30%, 70% and 100% ethanol and finally immersing in xylene and mounting in DPX. Quantification of the stained areas was carried out using a semi-automated method on an image analyser (Seescan plc - Salandra system). Areas of dermis and epidermis were traced on the video screen and numbers of positively stained cells and area of positive staining was calculated per mm^2 dermis or epidermis, based on at least three fields of view at x 10 magnification (mean of three separate measurements) depending on the size of the section. Expression of adhesion molecules was calculated as area stained per area measured, as positively staining cell numbers were difficult to determine. CD3$^+$ and CD45RO$^+$ T cells and CD1a$^+$ LC were expressed as number of cells stained per area measured. Standard deviation from the means was calculated in all cases and statistical analysis was performed using the Mann-Whitney U test. Non-specific staining was not observed in any of the controls which were incubated with diluent instead of primary antibody or with irrelevant antibodies.

Table I

Antibody	Source	Product Code	Dilution
anti-CD3	Dako	M 835	1/20
anti-CD1a	Dako	M 721	1/20
anti-CD45R0	Dako	M 742	1/200
anti-ICAM-1	British Biotech	BBA 3	1/200
anti-ELAM-1	British Biotech	BBA 1	1/100

Results

On histopathology, the morphological features of the wart specimens were confirmed. None showed evidence of specific inflammatory changes or resolution.

Adhesion molecule expression

The expression of ICAM-1 and ELAM-1 in the dermis of normal skin, warts and psoriasis is shown in *Fig. 2*. Positive staining is expressed as area stained (µm^2) per area measured (mm^2). Normal skin demonstrated small stained areas of ICAM-1 and ELAM-1 on vascular endothelial cells *(Fig. 1a)* which was not observed elsewhere (with a range of 2 ± 1µm^2 to 4 ± 1µm^2 for ICAM-1 and 0 to 3 ± 0 µm^2 for ELAM-1). In contrast, warts showed a significant increase in the areas of ICAM-1 and ELAM-1 stained *(Figs. 1b and 1c)*. The areas stained ranged from 13 ± 4 µm^2 to 48 ± 17µm^2 for ICAM-1 and 12 ± 8µm^2 to 35 ± 17 µm^2 for ELAM-1.

Positive staining for both ICAM-1 and ELAM-1 was in longitudinal or circular strands typical of vascular endothelium in the

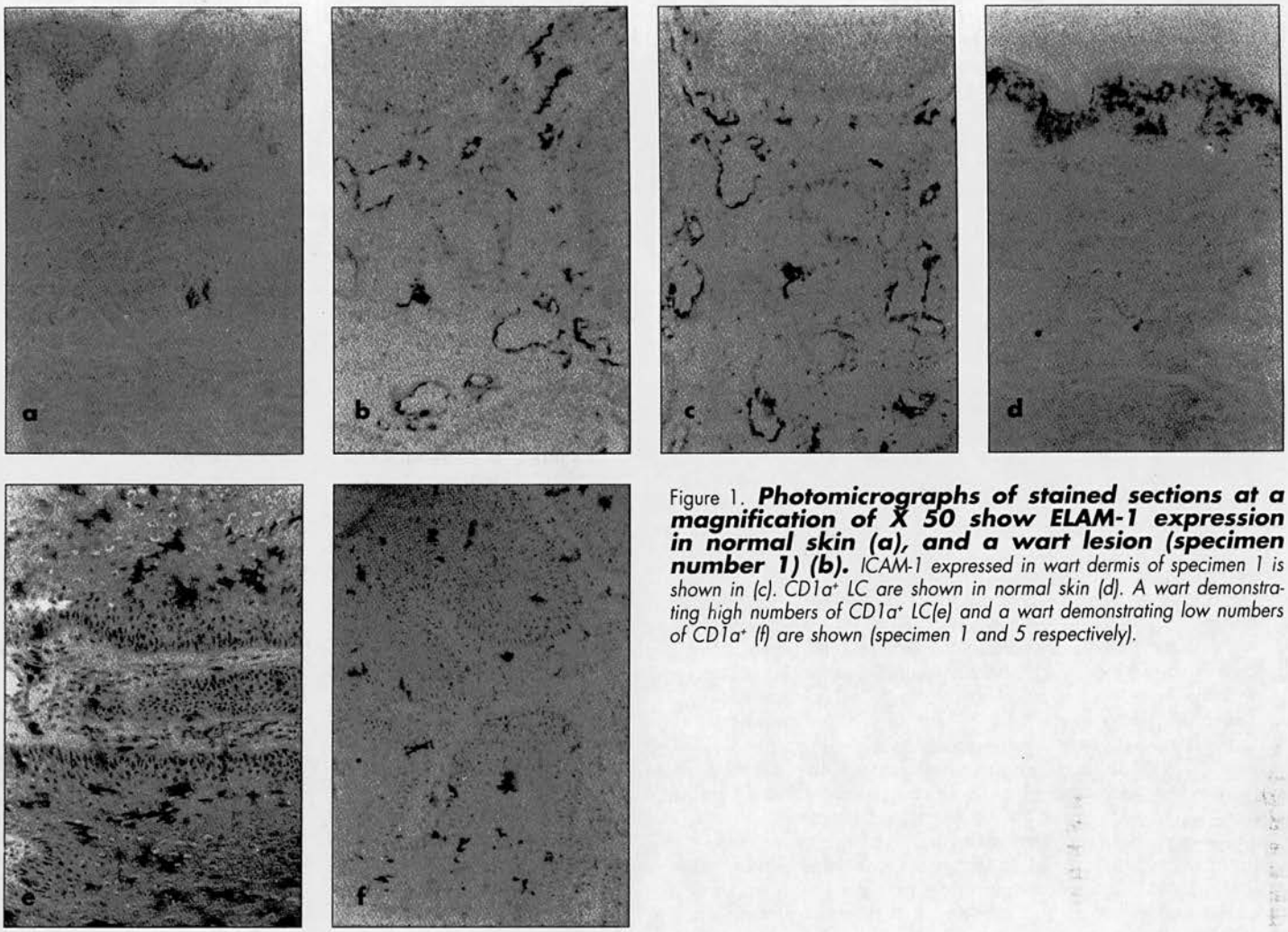

Figure 1. ***Photomicrographs of stained sections at a magnification of X 50 show ELAM-1 expression in normal skin (a), and a wart lesion (specimen number 1) (b).*** *ICAM-1 expressed in wart dermis of specimen 1 is shown in (c). CD1a⁺ LC are shown in normal skin (d). A wart demonstrating high numbers of CD1a⁺ LC(e) and a wart demonstrating low numbers of CD1a⁺ (f) are shown (specimen 1 and 5 respectively).*

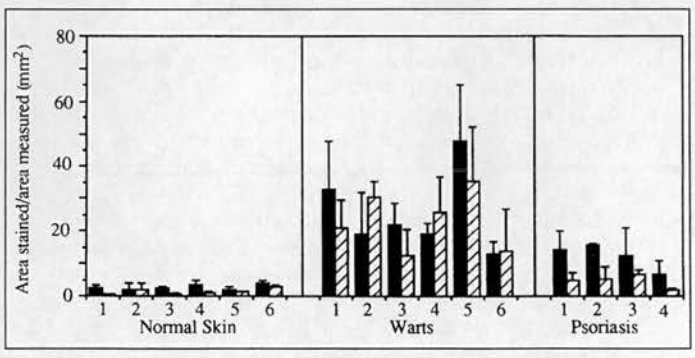

Figure 2. ***Expression of adhesion molecules in the dermis of 6 normal subjects, 6 with warts and 4 with psoriasis.*** *Bars depict standard deviation from the mean. ■ = ICAM-1, □ = ELAM-1. The area of ICAM-1 and ELAM-1 staining in the dermis was significantly increased (p < 0.005 in both cases) in wart dermis compared with the dermis of normal skin. The ICAM-1 and ELAM-1 staining was also significantly greater in the dermis of psoriatic skin compared with normal dermis (p < 0.05 in both cases). No significant difference was observed in the expression of these adhesion molecules in the dermis of warts and psoriatic skin.*

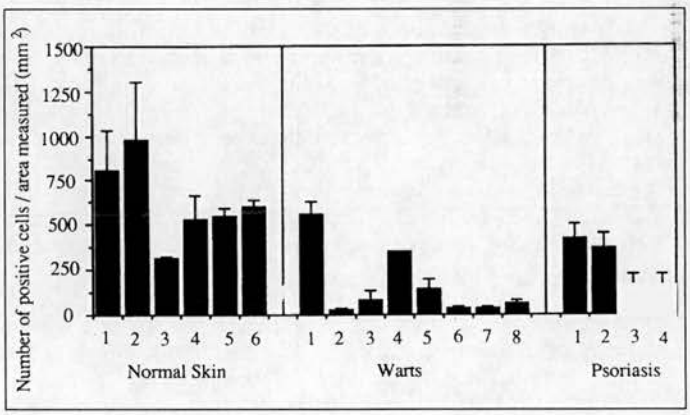

Figure 3. ***CD1a⁺ Langerhans' cells in the epidermis of 6 normal subjects, 8 with warts and 4 with psoriasis.*** *Wart patients 1-5 had papilliferous lesions, patient 6 had plantar warts and 7 + 8 had common hand warts. Bars depict standard deviation from the mean. Numbers of CD1a⁺ LC were significantly greater in the epidermis of normal skin compared with wart epidermis or with psoriatic skin (p < 0.05 in both cases). No significant difference was observed between the numbers of LC in the psoriatic epidermis and wart epidermis.*

dermis. The warts were more highly vascularized than the normal skin, and a high proportion of the vascular endothelial cells was positive in the warts. The dermis of psoriatic lesions also expressed ICAM-1 and ELAM-1 on the vascular endothelium with significantly higher staining than in normal skin. No difference was seen between the expression of adhesion molecules in the wart dermis and psoriatic dermis. ICAM-1 positive keratinocytes were not detected in the epidermis of any sections tested in this study, although ICAM-1 positive keratinocytes were detected in the epidermis of one patient with chronic atopic eczema (not shown).

Langerhans' cells

CD1a (T6) positive cells with dendritic morphology typical of LC were strongly expressed in the epidermis of normal skin *(Fig. 1d)*. *Fig. 3* shows quantification of CD1a expression as number of positive cells per total area of epidermis measured (mm^2). Stained cells were observed throughout the depth of the epidermis of normal skin, the number of positive cells varied from 314 ± 8 to 981 ± 318 per mm^2. In contrast the warts contained variable numbers of $CD1a^+$ LC. Warts 1 and 4 showed CD1a expression with a wide distribution throughout the epidermis *(Fig. 1e)*; staining was also observed in the papillary dermis although these cells had a rounded morphology, and some $CD1a^+$ cells with dendritic morphology were seen around the blood vessels in the dermis. Warts 3 and 5 showed a much sparser staining of CD1a, although it was still evenly distributed throughout the epidermis *(Fig. 1f)*. The other four warts had a few LC in the epidermis with CD1a staining only in the basal and mid-epidermis; there was no detectable staining in the dermis. The CD1a expression in the epidermis of all 8 warts was significantly different from CD1a expression in the epidermis of normal skin. CD1a expression in the epidermis of psoriatic lesions was also significantly decreased in comparison with normal epidermis (*Fig. 3*). No significant difference was observed between the CD1a expression in the epidermis of patients with warts and with psoriasis.

T cells

Fig. 4 shows the numbers of $CD3^+$ and CD45 RO^+ T cells per mm^2 of dermis and epidermis. Normal skin had little or no mononuclear cell infiltrate into the dermis or epidermis. $CD3^+$ T cells in the dermis were in the range 26 ± 6 to 140 ±88 per mm^2 and were not significantly different from the numbers found in the epidermis (0 to 166 ± per mm^2). Cells bearing the CD45R0 marker were present at similar frequencies to the CD3 marker in both the dermis and the epidermis, suggesting that most of the T cells present were of the memory phenotype. Both cell markers were observed in the perivascular area of the dermis. The $CD3^+$ infiltrate varied in the wart patients from a minimal dermal infiltrate (53 ± 36 cells/mm^2) to a large dermal infiltrate (654 ± 242 cells/mm^2). Many of these T cells were CD45 R0 positive. Interestingly, the numbers of $CD3^+$ and $CD45R0^+$ T cells decreased significantly in the epidermis of wart patients compared with the dermis ($p < 0.005$, Mann-Whitney U Test). Psoriatic lesions had comparable numbers of T cells infiltrating the dermis as the warts but more in the epidermis *(Fig. 4)*. The wart epidermis was more comparable to normal epidermis in terms of intraepithelial T cell numbers.

Discussion

As warts display increased dermal vascularity and epidermal hyperplasia in comparison with normal skin, biopsies of involved psoriatic lesions, representing a persistent inflammatory skin condition were chosen as appropriate controls. T cells and LC were enumerated per mm^2 of cross-sectional area in order to remove variations resulting from differences in epidermal thickness. This procedure has been recommended previously for the quantification of LC [19].

This study showed that the low numbers of intraepidermal T cells in cutaneous wart lesions were comparable with normal skin. However, the numbers of T cells in the dermis of warts were comparable with numbers found in psoriatic dermis and adhesion molecule expression was up-regulated. Unlike normal skin, the LC found in the epidermis of 6 out of 8 warts were very sparse.

Adhesion molecules and T cells

Most of the endothelial cells present in the dermis of warts expressed ICAM-1 and ELAM-1 at high levels. It has been suggested that ELAM-1 and other selectins initiate the adhesion of resting T cells and neutrophils to activated endothelium. The CLA marker on T cells has been proposed as the ligand for ELAM-1 [17], and this interaction may represent a skin-specific homing mechanism, to traffic memory T cells to cutaneous sites. The results presented in this study suggest that ELAM-1 may be important in the initiation of adhesion of memory T lymphocytes to vascular endothelium in the wart dermis. The upregulation of integrins on the T cell surface mediates strong adhesion to the endothelium via VLA-4 and VCAM-1, LFA-1 and ICAM-2 and LFA-1 and ICAM-1 interactions, which then proceeds to egression into the tissues. Only the expression of ICAM-1 has been considered here although ICAM-2 was expressed on the vascular endothelium of wart dermis (data not shown). The expression of ICAM-1 at high levels in the dermis shows that this adhesion molecule also has a role in the homing of T cells into the dermis of wart lesions. ELAM-1 and ICAM-1 were both expressed at low levels in the dermis of normal skin, which may indicate that they play a role in the normal surveillance of cutaneous sites.

ICAM-1 was not observed on keratinocytes in the epidermis of warts. This could be one explanation of why there were low numbers of T cells in the epidermis of warts, as the $ICAM\text{-}1^+$ KC interaction with $LFA\text{-}1^+$ T cells has been suggested as having a role in the epidermotropism of T cells. Alternatively the repertoire of integrins expressed on the local T cells may be abnormal. The epidermal keratinocytes in psoriatic lesions were also negative for ICAM-1, although many intraepithelial T cells were found, which indicates that there is most likely another mechanism for this movement into the epidermis, for example epidermal derived cytokines.

ICAM-1 has been demonstrated in the keratinocytes of psoriatic lesions [12-14] but Sheynius *et al.* found only 45% of patients tested demonstrated this [20]. ICAM-1 has been seen on the keratinocytes of laryngeal HPV-induced lesions but only when intensely infiltrated [9]. This may suggest that ICAM-1, although it is involved in epidermotropism of T cells, may not be the initiator of such a movement. Indeed in one study, normal skin was found to contain some epidermal T cells which were not localised to $ICAM\text{-}1^+$ keratinocytes [21]. Other candidates for the movement of leukocytes into the epidermis are cytokines such as IL-8 and monocyte chemotactic and activating factor, or the adhesion molecule LFA-3, which is present on epidermal LC and keratinocytes of normal skin

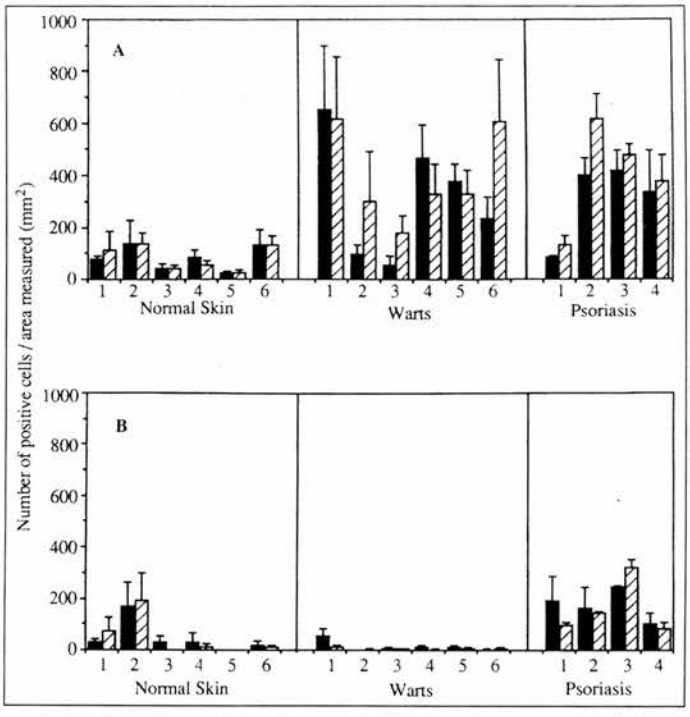

Figure 4. ***CD3⁺ ■ and CD45RO⁺ ▨ phenotypes in the dermis (A) and epidermis (B) of 6 normal subjects, 6 with warts and 4 with psoriasis.*** *Significantly greater numbers of T cells (CD45RO⁺ and CD3⁺ cells) were seen in the dermis of warts compared with normal dermis ($p < 0.05$ in both cases). No significant difference in the numbers of T cells was seen in the epidermis of warts and normal skin. Numbers of T cells in the dermis were significantly greater in psoriatic skin than in normal skin ($p < 0.05$ for both CD3⁺ and CD45 RO⁺ cells). T cell numbers were significantly greater in psoriatic epidermis as compared with wart epidermis ($p < 0.05$ for both CD3⁺ and CD45RO⁺ cells).*

and whose ligand is CD2 on T cells. However, it has been claimed that the ICAM-1 – LFA-1 pathway is most important in the adhesion of keratinocytes to T cells, at least *in vitro* [22].

Langerhans'cells

Six out of 8 of the warts in the present study showed a reduction in LC numbers in the epidermis compared with normal skin. Both of the warts which did not have reduced numbers were facial and papilliferous but the others of the same clinical type and from the same site had low counts. Chardonnet *et al.* [3] using a large number of warts from different anatomical sites, have shown a similar picture with many, but not all lesions containing lowered numbers of LC or none. This did not depend on the clinical type of papilloma but there was some indication that the location of the wart could influence the number of LC to some extent, with none being found most frequently in plantar warts (47%). It was demonstrated that depletion of LC in the epidermis correlated with the presence of HPV antigen. Detection of HPV antigen or typing of HPV was not included in the present study. Our control skin, normal and psoriatic, was taken from the forearm of volunteers due to the ethical difficulty of biopsying the face. Berman *et al.* [23] gave evidence of little difference in the density of LC in different anatomical sites, the head and neck having a mean density of 489 LC per mm^2 and the forearm having a mean density of 560 LC per mm^2.

We have no clinical or histopathological evidence that the warts, which showed no decrease in LC, were regressing. An increase in LC numbers has been observed in regressing warts and it has been suggested that LC play a role in this regression [4, 8, 24] but factors triggering the repopulation of the epidermis with LC have not yet been defined. Regressing plane warts show many LC in the dermis and epidermis with T cells observed adjacent to LC, and lymphocytes next to damaged keratinocytes in the epidermis [8]. This suggests a specific cell mediated immunity against virally infected keratinocytes.

It has been reported that non-viral skin tumours show an increase in LC numbers in the epidermis [25, 26] which could mean that HPV may reduce LC numbers specifically. However, in other studies, a reduced density of CD1a⁺ cells was found in such tumours [27-29]. It is possible that different HPV types may have differing immunogenicities which could affect the numbers of LC present. It still remains to remains to be shown whether HPV infects LC and has a direct cytotoxic effect on the numbers present in the epidermis. The reduction of LC in the epidermis of many skin warts may promote tolerance to viral antigens at this site and contribute to the persistent nature of these lesions. ■

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Reprints: M. Norval

Key words: adhesion molecules, cutaneous warts, human papillomavirus, immunohistochemisty, Langerhans' cells, T cells.

Acknowledgement

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ADHERENCE OF ACTIVATED T CELLS TO CUTANEOUS WARTS

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It has been shown previously that adherence of activated lymphocytes to both the dermis and epidermis of psoriatic skin is increased compared with normal skin, and the adherence is dependent on LFA-1 expression on lymphocytes and ICAM-1 expression in the skin. In this study adherence of activated lymphocytes to cutaneous warts was studied using a frozen section adherence assay. No increase in adherence was found in either the dermis or the epidermis of warts in comparison to normal skin. The cells which adhere were predominantly T cells ($CD3^+$) which were LFA-1^+ ($CD18^+$) . Previous work has demonstrated an increase in ICAM-1 expression and increased T cell numbers in the dermis of warts but no expression of ICAM-1 and reduced T cell numbers in the epidermis compared with normal skin. Thus, human papillomavirus infection of keratinocytes does not lead to the up regulation of ICAM-1 in the epidermis, which may be partly responsible for the poor influx of T cells into the lesion *in vivo* and the poor adherence *in vitro*. Reduced adherence to the dermis is not be explained by the lack of ICAM-1 expression, perhaps an indication for the involvement of other adhesion molecules or cytokines in T cell trafficking into the dermis underlying the wart.

25.

HUMORAL IMMUNITY TO *MALASSEZIA FURFUR* SEROVARS A, B AND C IN PATIENTS WITH PITYRIASIS VERSICOLOR AND CONTROLS.

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The aim of this study was to determine whether patients with pityriasis versicolor exhibited different humoral immune responses to *M.furfur* serovars A, B and C when compared to age and sex matched controls. A Transferable Solid Phase ELISA was used for the measurement of total immunoglobulins, IgM, IgA and IgG (Cunningham *et al.* Br J Dermatol 1992;127:476-481). A modification of this method was used to determine the titres of IgG subclasses specific to *M.furfur* serovars A, B and C (Fruin *et al.* Br J Dermatol 1992;127:433).

The mean reciprocal $\log_2$ antibody titres were analysed by Analysis of Variance and individual means compared using the T-method. Titres of total immunoglobulins (range 6.2-8.2), IgM (range 3.9-4.6) and IgG (range 4.8-8.4) did not vary significantly either between patients and controls or between serovars. However, titres of IgA were lower to serovar C in both the patient and control groups (2.4 and 3.2 respectively) than serovar A (6.1 and 6.6) or serovar B (5.0 and 6.2).

The titres of IgG subclasses varied between the subclasses, with generally lower titres of IgG_3 (3.4) compared to $IgG_{1,2,4}$ (4.4, 5.4 and 4.5 respectively).

In conclusion, although the distribution of serovars differs over the body, they were all presented to the immune system and elicited similar levels of primary and secondary responses. The production of IgG subclasses 1-3 in patients and controls suggests that both protein and carbohydrate antigens are present on *M.furfur*. The relative enrichment of IgG_4 indicated chronic antigen exposure in both groups. Thus, this study did not demonstrate any significant differences in humoral immunity to *M.furfur* serovars A, B and C in patients with pityriasis versicolor when compared to normal controls.

26.

EPIDERMAL IMMUNITY: INFLUENCE OF HLA-DR$^+$ AND CD1a$^+$ EPIDERMAL SUBPOPULATIONS ON THE STIMULATION OF LYMPHOCYTES

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We examined the importance of CD1a$^+$ HLA-DR$^+$ Langerhans cells and CD1a$^-$ HLA-DR$^+$ epidermal cells for the stimulation of allogeneic and autologous T cells.

Mononuclear leukocytes isolated from the peripheral blood of 7 healthy individuals and 7 patients with psoriasis were enriched to 96% by passage over nylon wool columns. Epidermal cells were isolated from the roofs of suction blisters by trypsinization and depleted of CD1a$^+$ and HLA-DR$^+$ cells by immunobeads. Cell enrichement and depletion were ascertained by FACS analysis. $1x10^5$ enriched T cells were incubated for 6 days with $1x10^4$ allogeneic and autologous epidermal cells. Cell proliferation was measured by ^{3}H-TdR uptake.

We found no significant difference between healthy controls and patients with psoriasis. In both groups depletion of CD1a$^+$ epidermal cells had no significant effect on stimulation of T cells, both in the allogeneic and autologous system. Compared to CD1a$^+$ cells, depletion of HLA-DR$^+$ epidermal cells further significantly diminished T cell proliferation in controls and patients.

We conclude that HLA-DR$^+$ epidermal cells are more important than CD1a$^+$ Langerhans cells for stimulation of both allogeneic and autologous T cells in normal controls and patients with psoriasis. Moreover our results indicate that there exists a CD1a$^-$ HLA-DR$^+$ epidermal cell population capable to stimulate T cells.

27.

CYTOKINE PRODUCTION AND T CELL PROLIFERATION IN PSORIASIS: ALTERATION OF CD4+ LYMPHOCYTES IN THE AUTOLOGOUS MIXED LYMPHOCYTE REACTION

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We examined the importance of CD8+ and CD4+ lymphocytes for the stimulation of autologous mixed lymphocyte cultures and autologous mixed lymphocyte epidermal cell cultures in patients with psoriasis and healthy controls. Furthermore, we investigated the influence of CD4+ cells on the production of TNF-α, IL-6 and IFN-γ.

Mononuclear leukocytes were isolated from the peripheral blood of 7 patients with psoriasis and 7 healthy controls by density gradient centrifugation. T lymphocytes and non-T lymphocytes then were enriched by passage over nylon wool columns. CD4+ and CD8+ cells were positivly selected by immunobeads. Cell enrichement and depletion were ascertained by flow cytometry. Epidermal cells were isolated by suction blister technique and trypsinization. $1x10^5$ CD4+ cells, CD8+ cells or unseparated T cells were incubated for 6 days with $1x10^4$ non-T lymphocytes or epidermal cells. Cell proliferation was measured by ^{3}H-TdR uptake. Cytokine concentration was determined in cell culture supernatants by ELISA technique.

Compared to cell reactions with unseparated T cells, cultures with CD4+ cells showed in healthy individuals a significantly ($p = 0.04$) higher proliferation in both autologous mixed lymphocyte cultures and lymphocyte epidermal cell cultures. By contrast, the proliferative response with CD4+ cells was not enhanced in patients with psoriasis. Furthermore, CD4+ cells clearly increased IL-6, TNF-α and decreased IFN-γ production in autologous mixed lymphocyte epidermal cell cultures in patients with psoriasis but not in healthy controls. In the autologous mixed lymphocyte reaction with CD8+ cells we found a significantly diminished cell proliferation compared to unseparated T cells only in psoriatics but not in healthy individuals.

These findings indicate an altered CD4+ cell function in psoriasis resulting in diminished cell proliferation and enhanced cytokine secretion in autologous mixed lymphocyte cultures and autologous mixed lymphocyte epidermal cell cultures.

28.

LOCAL IMMUNE RESPONSES IN CUTANEOUS WARTS

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Cutaneous warts, caused by infection with human papillomavirus (HPV) frequently persist for months or years; it is not clear what aspects of the immune system are most important in their control and resolution. The present study concentrated on an immunocytochemical analysis of Langernans' cells and T cells in cutaneous warts, together with an assessment of adhesion molecule expression ICAM-1 and ELAM-1). Comparisons were made throughout with skin from normal subjects and from patients with psoriasis. There was an increase in numbers of CD3$^+$, CD45RO$^+$ T cells in the dermis of warts compared with normal skin. In addition ICAM-1 and ELAM-1 expression were increased in the dermis of warts compared with normal skin which may be responsible for the influx of T cells into this site. However, a large reduction of CD3$^+$ CD45RO$^+$ T cells was seen in the epidermis of warts compared with normal skin, and epidermal ICAM-1 was not observed in warts. In 75% of the warts studied epidermal Langerhans' cell numbers were reduced in comparison with normal or psoriatic skin. Thus, a decrease in Langerhans' cells and a lack of adhesion molecule expression in the epidermis of warts may lead to a reduced influx of T cells into the lesion itself, although T cells are evidently present in the dermis underlying the infected site. It is possible that these local effects are induced by factors produced from keratinocytes as a result of HPV infection.